CALCIUM CARBONATE AS A DIETARY SUPPLEMENT:

ITS EFFECTS ON SOFT TISSUES, WITH SPECIAL REFERENCE TO ERYTHROPOIESIS

By

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PREFACE

This thesis describes some unexpected results obtained when a standard diet for breeding mice was supplemented with calcium carbonate at rates up to 2 per cent. The abnormalities observed included profound anaemia in both dams and their litters, and impaired growth and reproductive efficiency; in addition to the anaemia, young animals also exhibited thymus atrophy, hyperlipaemia, and fatty changes in the liver. The cause - or, at least, the major cause - of these disorders has proved to be an induced deficiency of iron, which can be largely overcome by feeding an iron supplement.

The work is presented in three Parts, with, in addition, Technical, Bibliographical and Tabular Appendices. Part I is introductory, and contains a description of the abnormalities caused by dietary supplements of calcium carbonate, together with details of the experiment in which they were first recognised. The major portion of the experimental work and argument is included in Part II. This Part begins with an appraisal of the probable importance of the observations, and proceeds to the formulation of a plan of research designed to elucidate the mechanism at work. The anaemia appears to be dyshaemopoietic in origin, and, after a survey of normal and abnormal erythropoiesis, its likely causes are listed. An experiment shows that a supplement of iron not only improves the blood haemoglobin concentration but also largely prevents the other abnormalities, and further evidence that calcium carbonate interferes with iron availability is adduced from later experiments. It is also shown that restoration of the calcium : phosphorus ratio, effected by means of a supplement of inorganic phosphate, does not abolish the deleterious effects of calcium carbonate.

Further work goes on to show that neither copper deficiency nor pyridoxin deficiency can have played any important part in causing the anaemia. However, supplements of cobalt or of dried thyroid (although not of iodine) increase the

haemoglobin level, and the effects of both are affected by the presence of calcium carbonate.

Following discussion of the other abnormalities observed in soft tissues, it is shown that in every case they could arise secondarily to anaemia. Nevertheless, other possible causes - including deficiencies of manganese, pyridoxin, thyroid hormone, choline and inositol - are suggested and investigated, but it is shown that none of these plays any part.

In Part III, the conclusion is reached that the effects on soft tissues which result from supplementing the diet with calcium carbonate can all be explained on the basis of an induced deficiency of iron, and that this is the major if not the sole cause of the entire syndrome. After reviewing the literature on calcium-phosphate-iron relationships, it is concluded that some important factor remains unrecognised, and suggestions are put forward to indicate the lines on which further investigations should proceed. Finally, the importance of the findings is discussed.

The first two experiments reported in this thesis were conducted jointly with Dr. Marion Richards, but our respective parts in the work remained distinct. The extents of our individual responsibilities have been clearly defined in the text, and only those parts of the work for which I was wholly responsible have been described in detail. I am also solely responsible for all aspects of the remainder of the experimental work, as well as for its interpretation, the conclusions drawn therefrom, the general argument of the thesis, and the reviews of the literature. All literature cited was consulted in the original, with the exception of a few papers which proved to be unobtainable; in these cases the sources of the references and information have been acknowledged.

I wish to thank Professor Drennan and Professor Boddie, of the University of Edinburgh, for their supervision of this work. I am also grateful to Dr. D. P. Cuthbertson, Director, The Rowett Research Institute, and to Dr. (now Professor) J. W. Howie, under whose immediate direction much of this work was done, for their advice and encouragement.

To Mr. A. W. Boyne, of the Rowett Research Institute, and to M. H. Quenouille, late of the University of Aberdeen, I am indebted for advice on the design of experiments and for statistical analyses of the data. Several other colleagues, whose contributions are acknowledged in the text, have also assisted me in various ways.

Finally, I wish to acknowledge the valuable technical assistance rendered by Mr. Edward Rattray, and also to express my appreciation of Miss Vivienne Farquhar's care of the experimental animals.

Aberdeen. ALASTAIR GREIG

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PART I

INTRODUCTION

PART I. - INTRODUCTION

A. THE BACKGROUND OF THE WORK

My interest in the subject which forms the title of this thesis arose from an observation made by my colleague Dr. Marion Richards. In order that the development of my studies may be made clear, it will be necessary briefly to explain the origin of Richards' work, and to refer in more detail to certain of her experimental methods and findings.

Richards' experiment derived from two separate lines of work pursued in the Rowett Research Institute. Richards herself had observed (Richards, 1945) that, in suckling rats, the effects of pyridoxin deficiency were accentuated when calcium carbonate was added to the diet. She had further noted that raising the calcium carbonate supplement from 0.54% to 1.62% retarded the growth of young pyridoxin-deficient weaned rats (Richards, 1949a). These dietary effects could not be explained at the time.

A little later, Howie and Porter (1950) had found that modifying a standard diet by the addition of casein 5% and calcium carbonate 1%, at the expense of whole wheat 6%, markedly reduced both the number and weight of progeny reared when the diet was fed to mated pairs of mice. The explanation of this result also was not obvious, although it was surmised that two factors could have contributed to it: either some unknown injurious influence exerted by the calcium supplement; or a deficiency of B vitamins (caused by the reduction in the intake of wheat, their main source in this diet) combined with an increased requirement (attributable to the increased protein intake); or possibly both.

These two sets of observations led Richards to investigate the effects,

on growth and reproduction in mice, of adding different levels of calcium carbonate to their diet, which was a slight modification of the well-known B diet of Sherman (Sherman and Muhlfeld, 1922; Sherman and Campbell, 1924; etc.); and at the same time - since this was also the standard diet employed by Howie and Porter (1950) - to attempt to discover which of the modifications made by these workers had been responsible for the effects they noted. This she did in Experiment 1*, in which I later joined. Those aspects of the experiment which have a direct bearing on my subsequent work must now be described at some length.

The B diet of Sherman consists of: ground whole wheat 2 parts and dried whole milk 1 part, with a supplement of sodium chloride equivalent to 2% of the weight of the wheat (i.e., 0.04 parts). The diet used by Howie and Porter (1950), and in this experiment by Richards, was first used by Schneider (1945) and is practically identical with Sherman's; it consists of ground whole wheat 66 parts, dried whole milk 33 parts, and sodium chloride 1 part. Thus the sodium chloride accounts for exactly 1% in this diet, compared with approximately 1.32% in the original B diet of Sherman. This modified Sherman B diet, which was used as the basal experimental diet, had a total calcium (Ca) content of 0.343%. When considering the calcium content of a diet, the level of phosphorus intake is also of interest and importance. This diet had a total phosphorus (P) content of 0.482%, thus giving a calcium : phosphorus (Ca : P) ratio of about 0.71 : 1.

As stated above, Howie and Porter's modifications of this diet led to a lowered wheat content, a raised protein (casein) content, and the inclusion of calcium carbonate. Accordingly, Richards imposed each of these three

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*This experiment has been reported in two published papers (Richards and Greig, 1952; Greig, 1952). Off-prints are included in the Appendix to this thesis.

alterations on the diet, separately and also in all possible combinations. This meant the use of eight diets. By using three levels of added calcium carbonate (0.5%, 1%, and 2%), as well as the control level of none, the number of diets now became 16. The reduction in wheat was accomplished by replacing 6 parts of it by maize starch, and the increase in protein by adding 5 (sometimes 6) parts of casein. The resulting factorial design is shown in Text Table 1, which also shows the approximate Ca : P ratios of each of the sixteen diets.

Sixty-four female albino Swiss mice, comprising 16 sets of four littermates reared on stock diet, were distributed equally among the 16 diets in this design. The distribution of littermates is shown in Text Table 2, and was such that the design remained balanced for the interactions between rows and columns.

The mice were introduced to the experimental diets when seven weeks old, and one week later were mated monogamously with males of the same age (but with the avoidance of brother-sister matings). The males were removed after 80 days, and time was allowed for any existing pregnancies to terminate and for the young to be reared. All litters were weaned at 21 days of age.

The criteria used in interpreting the breeding performance of each mated pair were based on the total number of pregnancies, the number of young born, and the number and weight of young reared. From these measurements certain ratios were derived. The results were given in detail by Richards and Greig (1952), and showed that breeding performance was significantly related to the level of calcium carbonate supplement in the diet. On the other hand, the differences associated with changes in the wheat and protein intake, and also the interactions between these dietary changes and the levels of supplementary calcium carbonate, were invariably small and did

TEXT TABLE 1.

Expt. 1. Design, and Ca:P ratios of Diets

(Ratio Ca:P shown as g. Ca per 1 g. P)

Modification	Nil (control)	0.5	1.0	2.0
	Added Calcium Carbonate (parts per 100 g. basal control diet)			
	Col. 1	Col. 2	Col. 3	Col. 4
Row 1 Nil (basal)	0.71	1.13	1.54	2.38
Row 2 Low wheat	0.74	1.17	1.61	2.48
Row 3 Low wheat-high protein	0.68	1.07	1.47	2.26
Row 4 High protein	0.66	1.05	1.43	2.20
Mean for Columns	0.70	1.11	1.51	2.33

TEXT TABLE 2.

Expt. 1. Distribution of Littermates within the Design

(Litters designated A-S, omitting I, J, and O)

	Col. 1	Col. 2	Col. 3	Col. 4
Row 1	EDGP	ABCQ	FMRS	HKLN
Row 2	ACFK	EMNP	GHLQ	BDRS
Row 3	BHMR	GKLS	CDEN	AFPQ
Row 4	LNQS	DFHR	ABKP	CEGM

not reach statistical significance for any measurement. Because of this latter finding, the data for all four rows could be considered homogeneous, and therefore, in estimating the effects of the various calcium carbonate levels in the columns, it was quite legitimate to group the four rows together. When this was done, it became clearly apparent that any effects caused by changes in the wheat and protein intakes were - if they existed at all - of negligible proportions compared with those resulting from the calcium carbonate supplements.

It will not be necessary for my purpose to reproduce the full data obtained in this experiment, but, because of their relation to my own later work, a summary of certain of the results of the breeding trial, grouped in the manner described above, is given in Text Tables 3 and 4. In some instances adjustment was made statistically to eliminate random effects, and in most cases this led to a substantial improvement in the regularity of the measurements within each treatment. Where this was done, only the adjusted means have been included in the Tables, together with the standard error of the differences between them.

The data show clearly that the highest level of supplementary calcium carbonate had markedly deleterious effects on the breeding performance of the animals to which it was fed. These effects were particularly noticeable on the rearing performance - the number weaned, the weight weaned and the survival rate being significantly poorer at this level than at any other. In almost every measurement column 3 fell intermediately between column 4 and columns 1 and 2, although its differences from these two columns did not attain an unequivocal level of statistical significance. In general, and especially with first litters (which were less variable than subsequent litters), the lowest level of supplement (column 2) appeared to

TEXT TABLE 3.

Exp. 1. Summary of Reproduction Results - 1st litters.

Measurement	Col. 1	Col. 2	Col. 3	Col. 4	Mean	S.E.±
Litters Born (No.)	16	16	16	15	15.8	
Litters Weaned (No.)	15	15	12	10	13.0	
Mice born/litter (No.) (Adjusted data)	7.8	8.1	7.2	6.6$^{\checkmark}$	7.4	0.66
Mice weaned/litter (No.) (Adjusted data)	5.2	7.2^{x}	4.9	2.4^{xxx}	4.9	0.86
Weight weaned/litter (g) (Adjusted data)	38.4	50.2	32.4	14.5^{xxx}	33.9	6.09
Mortality rate before weaning (%) (Adjusted data)	36	14	32	62^{xx}	36	11.2

^{x} Significantly different from all other columns ($P < 0.05$).

^{xx} " " " " " " ($P <$ or $\doteq 0.01$).

^{xxx} " " " " " " ($P < 0.001$).

$^{\checkmark}$ " " " column 2 ($P < 0.05$).

TEXT TABLE 4.

Exp. 1. Summary of Reproduction Results - full experimental period, all litters

Measurement	Col. 1	Col. 2	Col. 3	Col. 4	Mean	S.E.±
Totals (unadjusted data):						
Litters born (No.)	42	37	42	42	40.8	
Litters weaned (No.)	38	36	35	33	35.5	
Mice born (No.)	313	307	290	274	296	
Mice weaned (No.)	247	274	217	154	223	
Weight weaned (g.)	2065.0	2082.8	1739.1	1144.8	1757.9	
Means per mated female (adjusted data):						
Mice born (No.)	19.6	19.2	18.2	17.1	18.5	1.96
Mice weaned (No.)	16.4	17.8	14.6	9.6 xxx xx	14.6	1.83
Weight weaned (g.)	138.2	135.7	116.8	71.6	115.6	16.97
Mortality rate before weaning (%)	21	12	22	48 xxx	26	7.1

x Significantly different from all other columns ($P < 0.05$).

xx " " " " " " ($P <$ or $\doteq 0.01$).

xxx " " " " " " ($P < 0.001$).

/ " " " column 2 ($P < 0.05$).

give better results than no supplement at all (column 1), but only in one instance did the difference reach significant proportions.

The differences in numbers born followed the same pattern, but only with first litters was the calcium carbonate effect significant.

In previous experiments, Richards (1949a) had observed a reduction in the size of the thymus gland in pyridoxin-deficient rats receiving a high level of dietary calcium carbonate. Because of this, during the course of the present experiment she sacrificed a number of the young mice at weaning and examined their organs. Post-mortem inspection of the viscera showed that in many instances the thymus glands were very small, the hearts remarkably enlarged, and the livers pale and fatty in appearance. She began, therefore, to record the weights of these organs, and in addition she weighed spleen and kidneys. Before long it became apparent to her that small thymus glands and large hearts were associated with a high level of calcium carbonate in the mother's diet. It was at this stage that I was invited to collaborate by studying the pathology involved.

The results of my investigation (Experiment 1) are reported in the following pages.

B. PRELIMINARY STUDIES - EXPERIMENT 1

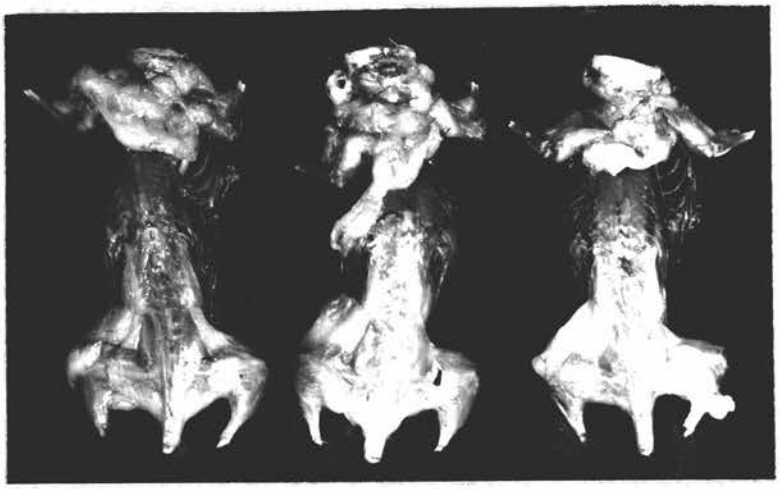
(i) Macroscopical Examination

Ante-mortem examination. At weaning age (21 days old) there were usually distinct and obvious differences between litters from different diet-groups.

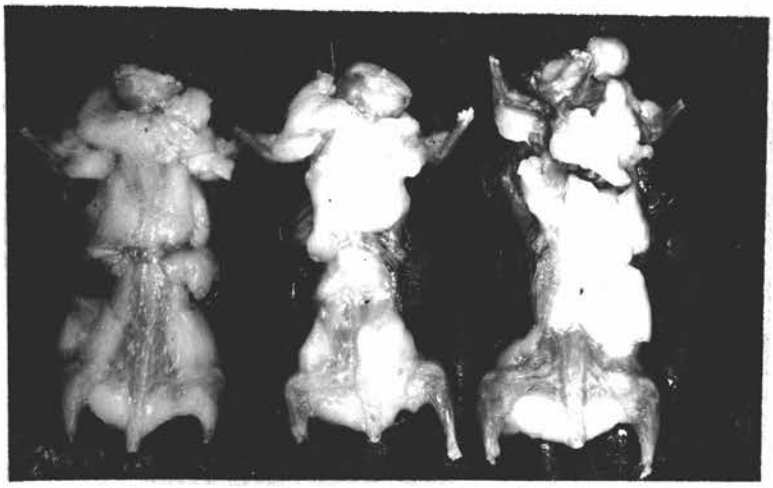
In general, weanlings from the high calcium carbonate groups were puny and ill-developed. This was evidenced not only by reductions - which were often quite remarkable - in their size and weight, but also by other features

FIGURE 1

Exp. 1. Deposits of subcutaneous fat on carcases of weanling mice



(a) Three weanlings from a low calcium carbonate diet-group.



(b) Three weanlings from a high calcium carbonate diet-group.

characteristic of poorly nourished young mice, such as a short, relatively wide head and a thin, straggly, lustreless fur.

Weanlings from groups with either no calcium carbonate in the diet, or only the lowest level, were in most cases strong, bright and lively, and readily nibbled their mother's food. If disturbed they bounded around the cage. On the other hand, those from the two groups with the higher levels of supplement remained huddled together in the nest, breathing rapidly, and often could scarcely be induced to move, far less to frisk. A number showed evidence of diarrhoea. These symptoms and modes of behaviour were all most pronounced in those weanlings whose mothers had been fed the highest level of calcium carbonate.

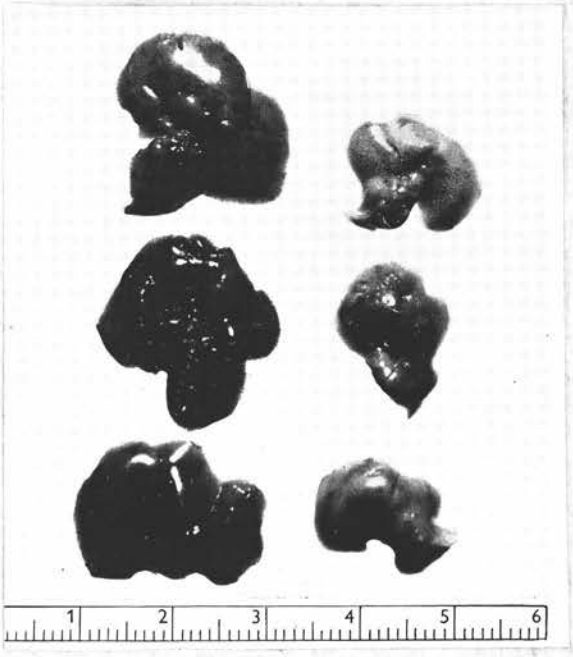
Post-mortem examination. The young mice were destroyed by placing them in a chamber into which was passed a current of coal-gas, and examinations were conducted very shortly after death on weanlings representative of the sixteen diet-groups. These examinations revealed distinct differences between the groups both in the carcases and in the size and appearance of certain organs.

The small, malnourished animals tended to be oedematous; though seldom severe, this feature was fairly constant. The normal fat depots were full in all animals. In the well-nourished weanlings the fat was firm and resembled lard in consistency, but in many of the others the depots were overloaded with large deposits of thin, oily fat, blanched and blubbery in appearance, which extended over the entire subcutaneous region of the dorsum of the trunk. In weanlings from groups on the highest calcium carbonate supplement these deposits were frequently quite massive (Fig. 1). The blood of these mice, instead of having the bright cherry red colour of more normal animals, was a dull, opalescent, pale creamy pink.

Of the visceral organs, the hearts, livers and thymus glands of weanlings

FIGURE 2

Exp. 1. Livers of weanling mice



(Left) low calcium carbonate diet-group.
(Right) high calcium carbonate diet-group. (Scale in cm.)

FIGURE 3

Exp. 1. Dissections of weanling mice

Showing body development and relative sizes of hearts and thymus glands in (left) low calcium carbonate and (right) high calcium carbonate diet-groups. (Scale in cm.)

from the high calcium carbonate groups presented the most striking changes. The hearts were pale and much enlarged, especially on the left side. The livers (Fig. 2) were very pale, dirty yellowish-brown in colour, friable and greasy; throughout the substance of these organs a fine mottling was very evident, corresponding with the lobular arrangement. The thymus glands of these animals were small and involuted, and often largely concealed by the enormous hearts (Fig. 3). The kidneys were rather pale but otherwise appeared normal. No other organs showed consistent changes, but occasionally a mild sub-acute enteritis was encountered in the lower portion of the ileum. The contents of the stomachs and other portions of the alimentary tract were variable in amount, but the organs were seldom quite empty.

These pathological features were found most consistently, and were most marked, in weanlings whose mothers had been fed the highest level of calcium carbonate supplement, but were also found to a slight degree, and occasionally to a moderate degree, in the unsupplemented groups and in the groups receiving the lowest level of supplement. No obvious correlation could be established between the pathological changes and the levels of wheat and of casein in the diets.

The following organs were removed from each weanling examined and placed in fixative (see the Technical Appendix, p. 257) for subsequent microscopical examination: heart, liver, thymus gland, spleen and kidneys.

As the breeding females became available at the end of the experiment, they also were killed and examined. In these animals, however, abnormalities were less pronounced, and their association with particular diets was much less obvious. Nevertheless, it was apparent that the carcases and organs of those dams which had been fed the higher levels of calcium carbonate usually exhibited greater pallor than those of the others, and their hearts often

appeared rather larger. The livers, although variable, were often fatty, some from the high calcium carbonate groups in particular being very severely affected. Thymus glands were of course all involuted in these adults, and the quantity and distribution of carcase fat presented no remarkable features except in a few animals which had failed to breed well; in them there were large accumulations of fat both within and without the normal depots.

Interpretation. Considered as a whole, the macroscopic findings strongly suggested the existence of a severe anaemia, associated particularly with diets containing the highest and the intermediate levels of calcium carbonate supplement. The immediate cause of the anaemia was not obvious, nor could it be decided at this stage whether indeed it was the primary disturbance or whether it was secondary to some other pathological condition. Certainly all the symptoms and morbid anatomy described above could be accounted for as sequelae of a severe degree of anaemia alone (this will be discussed later), but the existence of some other underlying cause or causes could not be ruled out.

As regards the classification of the anaemia, little could be inferred. The absence of marked jaundice or of internal bleeding suggested an interference with blood formation rather than some kind of abnormal blood destruction or loss, but the appearance of the bone marrow provided little help except that it excluded the possibility of aplastic anaemia. The marrows of all animals examined were of the rich red, highly active type normally found in young growing animals and in females recovering from the effects of an uninterrupted series of pregnancies and lactations.

As the macroscopical examination had revealed changes suggestive of anaemia, a study of the blood picture was clearly necessary. I therefore decided to make a haematological examination of all animals still remaining

in the experiment.

(ii) Haematological Examination

(a) Litters

At the time the blood studies commenced the breeding experiment itself had reached its concluding stages, the parent males having been removed from the cages some time previously. It was therefore not possible to obtain a complete and representative set of litters covering all the diet-groups. It will be recalled that after the removal of the sires from the cages existing pregnancies were allowed to terminate and the dams allowed to rear these final litters. Consequently it happened that in four instances, where a lactating mother had conceived again, two successive litters from one dam became available; in the majority of cases (36) one litter remained; but in 24 instances, where the dam had not again become pregnant, no litter at all could be examined. No first litters remained, so that the observations were confined mainly to second and third litters, with a few fourth litters (see Text Table 5).

Procedure and Methods. As each litter weaned, samples of blood were withdrawn under ether anaesthesia from every weanling in the litter, and the haemoglobin concentration of each sample was estimated by the method of Haldane (1901). After a few days, when 22 litters (covering 14 out of the 16 diet-groups) had been dealt with in this way, it became apparent not only that many of the litters were extremely anaemic, but that most of the lowest values had been recorded from weanlings whose mothers had been fed the highest level of calcium carbonate (i.e. from diets in column 4 of Text Table 1). The existence of anaemia had now been established, and consequently a more complete examination of the blood was thereafter undertaken with the object of obtaining some information on its characters. A stained film was

TEXT TABLE 5.

Exp. 1. The numbers of litters available for haematological examination.

The letters refer to the littermate grouping (see Text Table 2).

The base figures indicate the order of the litter, i.e. 2nd, 3rd or 4th.

Small letters denote that only Hb estimations were made; capital letters that the blood was examined in more detail.

	Col. 1	Col. 2	Col. 3	Col. 4
Row 1	p_2	c_2 A_3	m_3r_2 F_3M_4	$h_2k_3l_2$ N_4K_4
Row 2	k_3 F_3	m_2 N_3	$h_3l_3q_2$	s_2 $B_4D_3S_3$
Row 3	B_4H_3	$g_2k_2s_2$	n_2 C_3E_3	f_3 A_2
Row 4	n_3q_3 L_3N_4	r_2 F_3H_3	A_3	c_2e_3 G_3

Totals by litter order

	2nd litters	3rd litters	4th litters	Total	Stock diets (Nos. 56, 57 & 61)
Hb estimation only	14	9	0	23	5 (0, 0, 5)
More detailed examination	1	14	6	21	11 (5, 4, 2)
Total	15	23	6	44	16 (5, 4, 7)

Detailed blood examination

	No. of Experimental litters	No. of Stock litters
R.B.C.	21	11
W.B.C.	16	11
P.C.V.	13	11

prepared from the blood of each weanling, erythrocyte counts (R.B.C.) and leucocyte counts (W.B.C.) were made, and the percentage volume of packed red cells (P.C.V.) estimated in a haematocrit tube. (This latter determination could not be undertaken from the start, as the quantity of blood obtainable from a weanling mouse was insufficient to fill a Wintrobe haematocrit tube; capillary micro-haematocrit tubes had therefore to be obtained specially for this work.) Following the withdrawal of the samples, the animals were destroyed and passed to Dr. Richards, who, as described earlier, dissected out and weighed certain organs. These organs were returned to me for subsequent microscopical examination (see p. 19).

Sixteen weaning litters on three stock diets (Diets 56, 57 and 61) were similarly examined as extra-experimental controls.

The technical methods employed in carrying out these procedures, and the reasons for their choice, are discussed fully in the Technical Appendix (p. 249).

Results. Out of the 142 litters weaned in the experiment, haemoglobin estimations were performed on only 44 (totalling 182 weanlings) and of these no more than 21 (57 weanlings) were available for fuller examination. As explained above, the 44 litters comprised a mixture of second, third and fourth litters, and were from only 40 of the 64 dams. The material was thus very incomplete, although it happened that every diet was represented at least once (see Text Table 5).

Nevertheless, an attempt was made to analyse the haemoglobin figures. Diet-group means were computed by taking the mean for all the young in each litter, and then finding from these statistics the mean for all the litters in each diet-group. The small number and irregular distribution of the available litters meant, however, that there was insufficient information to warrant

weighting the means for either the number of young in the litter or the order of the litter (i.e. whether second, third or fourth). For the same reasons the accuracy of the comparisons could not be much increased by making use of the factorial design, although some correction was possible for variability attributable to littermate factors. The relatively small numbers also precluded consideration of each diet-group separately, but the overall effects of modifications in the dietary levels of wheat, casein and calcium carbonate could be determined by combining, as marginal treatment means, the values in each row and in each column. A summary of the results of this operation is given in Text Table 6. The standard error quoted therein is only approximate, being the mean of the individual correct standard errors; but the significance of large differences was tested by exact analysis. The full experimental data are contained in Appendix Table I.

Because of the incomplete and mixed nature of the material, highly significant differences could hardly be expected; but the results of comparing means by the t test were surprising. From Text Table 6 (see also Fig. 4) it can be seen that the mean Hb values for the first and second columns are significantly greater than the value for the fourth column ($P \ll 0.01$ and < 0.05 respectively). The value for the third column is intermediate. On the other hand, differences between the rows fail in every instance to reach statistical significance.

It is true that the marginal mean for row 1 is lower than that for any other row, and indeed its difference from that of row 4 approaches significance at the 5% level. However, it can be seen that the low marginal mean in row 1 is due very largely to the abnormally low value in column 1; while the mean for row 4 is the highest largely because of the abnormally high value in column 3. From Text Table 5 it can be seen that both these values are not really means for the diet-groups, as in each case only one litter was examined. Going more deeply into the matter, we find that the litter in row 1 column 1 was born of a mother in litter 'P', which is not represented elsewhere in the Table;

TEXT TABLE 6.

Exp. 1. Litters. Hb Treatment Means (g./100 ml.)

In some instances the marginal means have been adjusted statistically to correct for random variability.

The number of litters on each treatment is shown in Text Table 5. .

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean
Row 1	3.3	6.2	4.3	3.9	4.4
Row 2	8.3	3.0	5.5	4.1	5.3
Row 3	7.3	6.7	4.0	4.7	5.5
Row 4	7.2	6.7	7.7	3.1	5.9
Marginal Mean	7.0	5.8	4.9	3.9	5.3

Mean standard error of difference between marginal means = $\pm$ 0.76.

The mean for column 4 is significantly lower than those for column 1 ($P < 0.01$) and column 2 ($P < 0.05$).

TEXT TABLE 7.

Exp. 1. Litters. Marginal means of blood attributes

Attribute	Col. 1	Col. 2	Col. 3	Col. 4	Diet 56	Diet 57	Diet 61
Hb	7.0	5.8	4.9	3.9	12.0	9.6	9.3
R.B.C.	5.3	5.8	5.0	3.8	7.7	6.6	7.8
M.C.H.	11.1	10.8	10.2	10.4	15.4	14.9	14.6
P.C.V.	16.8	11.8	17.3	13.5	35.3	29.2	33.5
M.C.H.C.	33.5	35.0	31.3	29.9	33.5	33.9	32.7
M.C.V.	37.8	29.1	33.6	31.6	47.1	44.8	42.9
W.B.C.	1.50	0.80	0.82	0.73	1.41	1.56	0.95

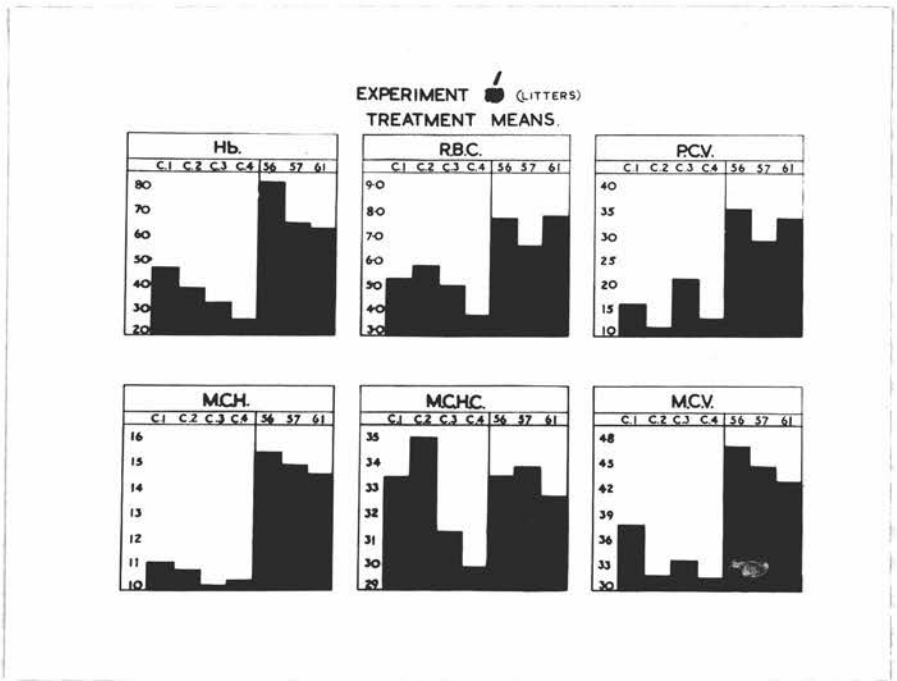
no comparisons are therefore possible. That in row 4 column 3, however, derives from litter 'A', which is represented in two other diet-groups (row 1 column 2, and row 3 column 4); from Appendix Table I it can be seen that in each case the litter from the 'A' mother had a much higher mean Hb than had its fellow (from 'C' and 'F' respectively). There are therefore important grounds for believing that the value in row 4 column 3 is artificially high, while no evidence can be adduced one way or the other in regard to that in row 1 column 1. In these circumstances, then, it would be highly dangerous to infer anything from such a difference in the absence of confirmatory or, at least, supporting evidence.

Turning now to the fuller investigations of the blood picture which were undertaken on 21 litters, it will be seen from Text Table 5 that some diets were not represented at all and a statistical analysis of the results was therefore out of the question. The best that could be done was simply to combine the raw data into marginal means, averaged over each column, without considering individual diet-groups separately and therefore not using diet-group means. Because of the unequal numbers of litters, this operation assumed that there were no real differences between the rows and also no row x column interactions. Marginal means for the rows were not computed, since no difference in Hb had been shown to exist between them. In any case, however, the distribution of these 21 litters is uneven (Text Table 5), there being in one row a preponderance of litters in columns 1 and 2, and in the others a preponderance in columns 3 and 4; this fact would certainly have affected the results to the extent that simple unweighted means would have had no meaning. Attention was therefore restricted to the columns.

The attributes examined were the erythrocyte count (R.B.C.), the leucocyte count (W.B.C.), the volume of packed erythrocytes (P.C.V.) and the erythrocytic indices (ratios) derived from Hb, R.B.C. and P.C.V., namely, the mean corpuscular haemoglobin (M.C.H.) - Hb : R.B.C.; the mean corpuscular haemoglobin concentration (M.C.H.C.) - Hb : P.C.V.; and the mean corpuscular volume (M.C.V.) - P.C.V. : R.B.C. The units of measurement used, and the

FIGURE 4

Exp. 1. Litters. Treatment means of Erythrocytic Attributes



methods of calculating these attributes, are described in the Technical Appendix (p. 257).

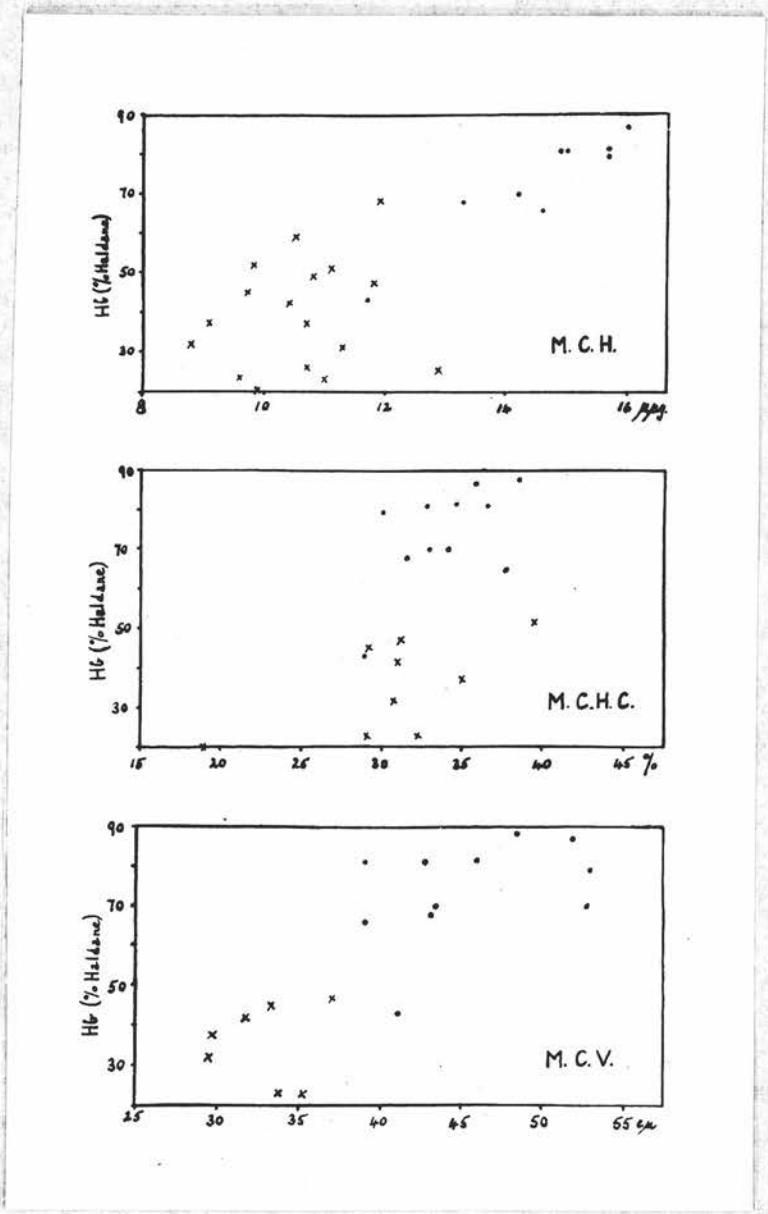
The overall marginal means are shown in Text Table 7, which, for the sake of comparison, also includes the mean values recorded from the three stock diets mentioned above. In Fig. 4 the marginal means of the erythrocytic attributes are presented in graphic form.

It must again be emphasised that these results should be viewed with great caution. Besides the introduction of a possibly large sampling error owing to the small number and irregular treatment distribution of the litters examined, the error inherent in the technical methods must also be remembered; the error in estimating R.B.C. (and, even more, ratios in which it is the denominator) is particularly important (Dacie, 1950). Although technical errors, being random errors, tend to cancel themselves when large numbers are being examined, there is less opportunity for cancellation when the sample is small.

Bearing these caveats in mind, then, the most that can be said from inspection of the Table and Figure is that, in general, there is a tendency for all blood attributes examined to become lower as the level of calcium carbonate in the mother's diet is increased; by tending to fall away from column 1 to column 4 the measurements thus follow the pattern set by Hb. The most notable exception to this general rule is found in columns 2 and 3 with the figures for P.C.V. and the ratios depending upon it, namely, M.C.H.C. and M.C.V. On further investigation, however, we find that it so happened that the seven litters on which the figures are based were actually the two most anaemic of all the litters in column 2 and the five least anaemic of all the litters in column 3 (see Appendix Table I); indeed the mean Hb for the column 2 pair was actually less than that of the five in column 3. This fact not

FIGURE 5

Exp. 1. Litters. Graphs of erythrocytic indices against haemoglobin concentration (litter-means)



o = stock litter

x = experimental litter

only explains the apparently anomalous behaviour of these three attributes in columns 2 and 3, but indeed gives support to the original interpretation.

There is a suggestion, then, although we must treat it with some reserve, that the anaemia may be hypochromic (low M.C.H.C.) and microcytic (low M.C.V.), and may be associated with a low M.C.H. If we assume that the anaemia is of the same aetiological origin in every column, and that its characters differ only in degree, we can obtain a more useful indication of its type by plotting the individual litter-means for these three attributes against the corresponding litter-means for Hb, and considering them irrespective of diet. When this is done (Fig. 5), the general tendency for a low Hb to be accompanied by a low M.C.H., a low M.C.H.C., and, possibly, a low M.C.V. becomes again apparent; but these correlations are in no instance strong.

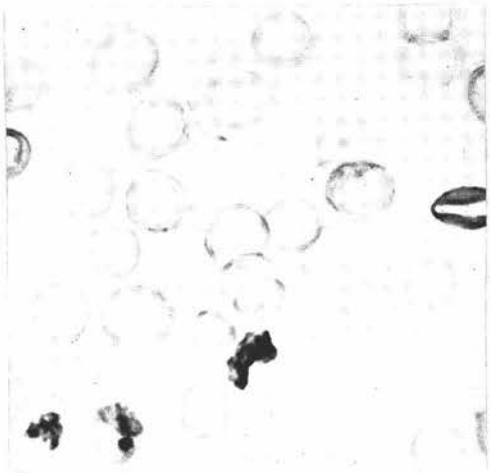
Much the most striking point about the results is that, in Fig. 5 as well as in Fig. 4 and Text Table 7, the litter-means of the stock litters are far higher than those of the experimental litters, for all attributes with the exception of M.C.H.C., where the effect is less obvious. This strongly suggests that, compared with the stock litters, litters from the experimental groups were affected with a microcytic anaemia which, when severe, was also hypochromic.

Microscopic examination of stained blood smears showed that the erythrocytes of anaemic weanlings were mainly hypochromic, sometimes severely so (Fig. 6). In addition to the polychromatic cells normally found in mouse blood, occasional orthochromatic cells, similar to those described by Price-Jones (1932), were also present and stood out in contrast. Anisocytosis was invariable and poikilocytosis frequent.

Blood films from the less anaemic groups sometimes presented similar abnormal features but to only a very slight degree. But when they were compared

FIGURE 6

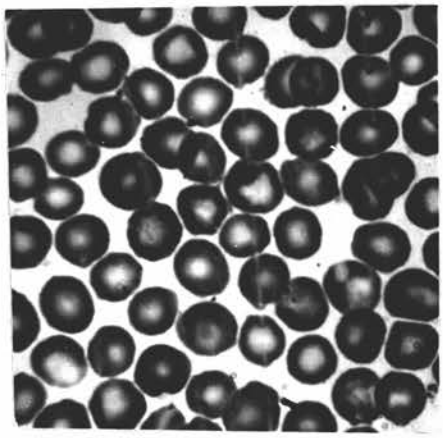
Exp. 1. Blood smear



Weanling from a high calcium carbonate diet-group. (Leishman; x 1800)

FIGURE 7

Exp. 1. Blood smear



Weanling from a low calcium carbonate diet-group. (Leishman; x 1800)

with films from the blood of stock-reared weanlings, the tendency towards a general microcytosis was quite evident (Fig. 7).

Conclusions. It has been shown beyond reasonable doubt ($P \leqslant 0.01$) that the addition of 2% of calcium carbonate to the B diet of Sherman caused anaemia in the weanling progeny of mated pairs of mice fed such a ration. It also seems quite likely that only 1% of calcium carbonate did so too, although to a less extent; but 0.5%, if it exerted any effect at all, did so to a significantly less degree ($P < 0.05$) than did 2% of the dietary supplement.

It would be dangerous to draw further conclusions of any but a tentative nature from these preliminary studies, but some evidence has been presented suggesting that the anaemia was hypochromic and possibly microcytic in type. Moreover, it seems fairly certain that all the experimental weanlings were anaemic compared with weanlings from stock diets; the anaemia was markedly microcytic, but not severely hypochromic unless the diet contained one of the higher levels of calcium carbonate.

(b) Dams

Procedure and Methods. All but six of the 64 dams were available for haematological examination. This was generally carried out on the same day as their final litter was weaned and examined, but in those instances where no litter was available the dams were examined as soon as it became apparent that they were not again pregnant. Thus, in addition to variations in the number of litters borne, another variable which could not be completely controlled was the number of days since suckling ceased. However, all animals examined were non-pregnant, and, with only one or two exceptions, had suckled a litter recently.

Determinations of Hb, R.B.C. and W.B.C. were made on all 58 dams. When capillary micro-haematocrit tubes became available, P.C.V. estimations were

also performed, 42 animals receiving the full examination. In addition, 9 dams on stock diet (the mothers of the extra-experimental litters referred to in the preceding sub-section) were similarly examined.

The procedures as regards technical methods and dissection were identical to those employed for the litters.

Results. In analysing the results for Hb, R.B.C., W.B.C. and M.C.H., the fact that only six observations were missing out of 64 enabled advantage to be taken of the statistical design. The arrangement of littermates made it possible to isolate and eliminate the component of variability attributable to litter-mate effects. But because 22 P.C.V. observations were missing, the design had to be treated simply as a randomised block for this attribute and for the erythrocytic indices depending on it, namely, M.C.H.C. and M.C.V.

The full data are given in Appendix Table II but for convenience they have been summarised in Text Tables 8-15, which give the diet-group means and marginal means for each attribute in turn, together with the standard errors of differences between means. The standard errors quoted are the mean standard errors in each case, but probabilities have been determined by reference to the exact standard errors. The summarised results in the Text Tables have been prepared from the adjusted data after elimination of littermate effects, and after estimation of missing observations* in order to make all comparisons orthogonal. For comparative purposes, mean values obtained from the animals on stock diets have also been included in the Tables.

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*Missing observations were kindly estimated for me by Mr. M. H. Quenouille, at that time Lecturer in Statistics, University of Aberdeen. The method which he employed is described in the following extract from his report:

".....The missing observations were G(86), M(111), G(88), M(112), R(172), and L(108). First approximations to the estimates of these observations were obtained by constructing values which were comparable with the diet, calcium and litter means concerned. Analyses of covariance were then carried out on six dummy variables x_1, x_2, x_3, x_4, x_5, and x_6. The variable x_1 had the value 1 for G(86) and was zero elsewhere; x_2 had the value 1 for M(111) and was zero elsewhere; and similarly for the other dummy variables. In this way six regression coefficients were obtained which were used to correct the tables of means. In no case was the regression sum of squares significant and therefore second approximations for the estimates were not made....."

TEXT TABLE 8.

Exp. 1. Dams. Hb Treatment Means (g./100 ml.)

The means have been adjusted statistically to eliminate between-litter effects.

Four animals per treatment; six observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	14.19	14.61	13.85	12.71	13.84	
Row 2	15.33	12.70	13.68	12.96	13.66	
Row 3	15.21	14.33	15.16	12.99	14.43	
Row 4	15.36	14.49	13.36	11.84	13.76	
Marginal Mean	15.02	14.03	14.02	12.62	13.93	16.26

Mean standard error of differences between (Marginal means = ± 0.662; Tabular means = ± 1.146)

The mean of column 4 is significantly less than that of all other columns ($P < 0.01, < 0.05, < 0.05$).

TEXT TABLE 9.

Exp. 1. Dams. R.B.C. Treatment Means (millions/cu.mm.).

The means have been adjusted statistically to eliminate between-litter effects.

Four animals per treatment; six observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	10.2	10.4	11.0	10.8	10.6	
Row 2	10.1	9.5	10.8	11.3	10.4	
Row 3	13.1	10.8	12.2	10.0	11.5	
Row 4	11.4	10.5	9.9	9.7	10.4	
Marginal Mean	11.2	10.3	11.0	10.4	10.7	10.7

Mean standard error of differences between (Marginal means = ± 0.621; Tabular means = ± 1.076)

No significant dietary effects are evident in this measurement.

TEXT TABLE 10.

Exp. 1. Dams. M.C.H. Treatment Means (μμg.).

The means have been adjusted statistically to eliminate between-litter effects.

Four animals per treatment; six observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	13.9	15.0	12.5	11.3	13.2	
Row 2	15.5	13.4	12.9	11.5	13.3	
Row 3	12.4	13.2	12.6	12.8	12.7	
Row 4	13.9	13.9	13.5	11.9	13.3	
Marginal Mean	13.9	13.9	12.9	11.8	13.1	15.3

Mean standard error of differences between (Marginal means = ± 0.92; Tabular means = ± 1.59)

The mean of column 4 is significantly less than those of columns 1 and 2 ($P < 0.05$, < 0.05).

TEXT TABLE 11.

Exp. 1. Dams. P.C.V. Treatment Means (%)

Four animals per treatment; twenty-two observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	47	39	47	44	45	
Row 2	49	36	36	47	41	
Row 3	52	46	42	43	45	
Row 4	46	34	46	37	42	
Marginal Mean	48	40	44	42	43	46

Mean standard error of differences between (Marginal means = ± 3.01; Tabular means = ± 6.02)

No significant dietary effects are evident in this measurement.

TEXT TABLE 12.

Exp. 1. Dams. M.C.H.C. Treatment Means (%).

Four animals per treatment; twenty-two observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	31.9	35.7	29.7	28.9	30.7	
Row 2	30.8	34.9	40.0	26.5	34.1	
Row 3	31.9	31.9	34.6	31.7	32.3	
Row 4	43.2	43.2	34.2	26.7	36.8	
Marginal Mean	35.8	35.8	33.7	29.1	33.5	34.6

Mean standard error of differences between { Marginal means = ± 3.69; Tabular means = ± 7.38

No significant dietary effects are evident in this measurement, although there is a suggestion that the value in column 4 may be significantly lower than those in columns 1 and 2 ($0.1 > P > 0.05$, in each case).

TEXT TABLE 13.

Exp. 1. Dams. M.C.V. Treatment Means (cu.μ).

Four animals per treatment; twenty-two observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	45.9	38.6	42.6	38.9	42.2	
Row 2	53.2	36.9	33.4	43.5	40.9	
Row 3	35.5	45.2	36.9	40.4	40.2	
Row 4	39.6	32.2	44.3	41.5	40.0	
Marginal Mean	43.1	38.8	40.4	40.6	40.8	43.5

Mean standard error of difference between (Marginal means = ± 3.37; (Tabular means = ± 6.75

No significant dietary effects are evident in this measurement.

TEXT TABLE 14.

Exp. 1. Dams. W.B.C. Treatment Means (thousands/cu.mm.).

The means have been adjusted statistically to eliminate between-litter effects.

Four animals per treatment; six observations which were missing have been estimated statistically and included in the means.

	Col. 1	Col. 2	Col. 3	Col. 4	Marginal Mean	Stock diets
Row 1	1.9	4.1	2.0	7.4	3.9	
Row 2	2.9	1.6	2.8	2.3	2.4	
Row 3	2.3	3.3	2.6	3.1	2.8	
Row 4	2.9	2.4	2.4	2.0	2.4	
Marginal Mean	2.4	2.9	2.4	3.7	2.9	3.3

Mean standard error of differences between (Marginal means = $\pm$ 3.73
(Tabular means = $\pm$ 6.46

No significant dietary effects are evident in this measurement.

TEXT TABLE 15.

Exp. 1. Dams. Haematological Correlation Coefficients.

Correlation	r	P
Hb and R.B.C.	+0.460	<0.001
Hb and P.C.V.	+0.667	<0.001
Hb and M.C.H.	+0.531	<0.001
Hb and M.C.H.C.	+0.362	<0.05
Hb and M.C.V.	+0.254	>0.10
Hb and W.B.C.	+0.313	<0.05

It will be seen that in no instance do the differences between the rows reach significance; there is not even evidence of any consistency from one attribute to another. On the other hand, comparison between the columns shows that the mean Hb of animals on diets containing the highest level of calcium carbonate is significantly lower than that obtained at all other levels ($\underline{P} < 0.01$; < 0.05; < 0.05). The mean M.C.H. at the 2% level of added calcium carbonate is also significantly lower than at the 0 and 0.5% levels ($\underline{P} < 0.05$; < 0.05). There is also a suggestion that a similar relationship exists between the M.C.H.C. and the level of supplement ($0.1 > \underline{P} > 0.05$).

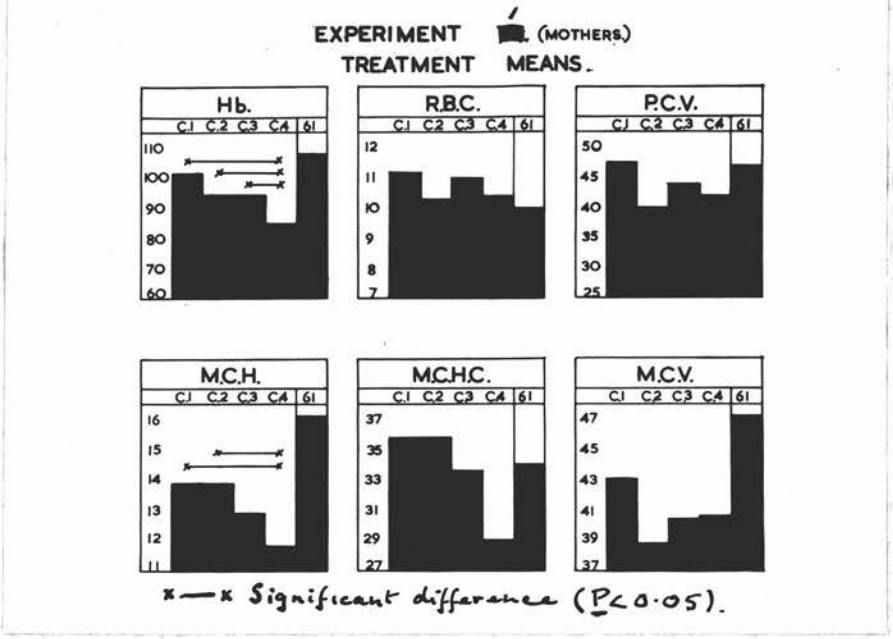
No significant effects are apparent on the other blood attributes, but at the same time it is remarkable that with every erythrocytic attribute the highest mean falls in column 1, and in most cases column 4 produced low values (Text Tables 8-13, and also Fig. 8). This latter point would be much more striking if it were not for the fact that the mean value for P.C.V. is extremely low in column 2, and consequently the M.C.V. in this column is also very low and the M.C.H.C. high. These exceptional values do not accord with the regular pattern of the values in other attributes.

> It is not easy to explain this low P.C.V. mean in column 2 except on the grounds of some large technical errors; and detailed examination of the figures shows that, in this column, only 7 out of a maximum 16 observations had been possible on P.C.V., so that the effect of any errors would be considerably magnified. Some difficulty had at first been met with in using the micro-haematocrit tubes, owing to badly fitting rubber pads and consequent leakage of the tube contents, and it so happened that four of these seven observations, including all the very low figures, were included in the first batch of recordings made with these new items of apparatus. It seems more than likely that this is the true explanation for the discrepancy.

As the individual results, with all attributes, overlap considerably from one column to another, a more accurate assessment of the type of anaemia might be gained by studying the animals as individuals instead of as groups.

FIGURE 8

Exp. 1. Dams. Treatment Means of Erythrocytic Attributes



(This procedure, of course, assumes that the anaemia is of the same type in every case.) Consequently, correlation coefficients between Hb - the measure of the degree of anaemia - and all other blood attributes were calculated from the data relating to each individual animal, irrespective of dietary treatments (Text Table 15). The most striking of these relationships is the strong positive correlation ($P < 0.001$) between Hb and P.C.V., and this is the more remarkable in view of the probability, mentioned above, that there were considerable technical errors in a few P.C.V. estimations. It can also be seen from the Table that Hb was strongly correlated with M.C.H. ($P < 0.001$), the relationship being practically linear. This means that in the anaemic animals the amount of haemoglobin carried by each erythrocyte diminished in proportion to the anaemia. The coefficients also confirmed that the blood of the anaemic dams (i.e. those with lower Hb values) had a lower M.C.H.C. ($P < 0.05$); that is to say, the anaemia was hypochromic. No significant correlation, however, could be shown between the Hb and M.C.V.; that is to say, the anaemia could not be shown to be other than normocytic.

Text Table 15 also shows that there was a significant ($P < 0.05$) correlation between Hb and W.B.C., indicating that the anaemia was accompanied by a leucopenia.

It is also interesting to compare these experimental animals with the extra-experimental breeding females on stock diets; these, although not littermates, were of the same strain and were kept under the same conditions. As can be seen from Text Tables 8-14, and from Fig. 8, even the best of the experimental diet-groups was anaemic compared with the controls. However, the R.B.C.s were generally similar with the result that the low M.C.H. of the experimental groups was associated more with a low M.C.V. than with a low M.C.H.C. - indeed the M.C.H.C. in columns 1 and 2 is higher than that of the stock mice. In this respect the comparison differs from the within-experiment

comparisons, where the anaemia was hypochromic.

In stained films made from the blood of anaemic dams, abnormalities were seen similar to those noted in the anaemic weanlings' blood; hypochromasia was never so severe, however, and poikilocytosis was unusual. Blood from the more normal experimental dams, when compared with blood from stock animals, showed no very marked differences on inspection although the average cell diameter appeared to be smaller.

Conclusions. The addition to the diet of calcium carbonate, at the rate of 2%, induced anaemia in breeding female mice. This anaemia was hypochromic in type, but did not appear to be markedly microcytic. It was accompanied by a leucopenia. Experimental animals in all groups had lower blood haemoglobin concentrations than had similar animals on stock diets; their blood was more microcytic, but except in the most anaemic group it was not more hypochromic.

(iii) Histological Examination

(a) Procedure and Methods

As mentioned on p. 10, certain organs of both litters and dams were retained for examination. These were the hearts, thymus glands, kidneys, spleens, and livers. Although sets of these organs were not available from all the experimental animals, there were sufficient to enable comparisons to be made satisfactorily and every diet-group was represented in both the litter and the dam series. A systematic examination of the bone marrow was not undertaken, but smears and sections were prepared from the femoral marrows of several representative weanlings and dams in each treatment column.

Paraffin sections of all these tissues, and also frozen sections of the livers, were prepared and stained as described in the Technical Appendix (p. 258).

(b) Results

Hearts. In both litters and dams from diet-groups containing the higher levels of supplement, the hearts exhibited marked hypertrophy of the individual muscle fibres and oedematous separation of the muscle bundles. In a number of the most anaemic weanlings from these groups, early degenerative changes were apparent in the heart-muscle cells (Fig. 9). Fatty degeneration was never seen, but in one particularly anaemic weanling numerous calcified areas were observed scattered throughout the muscle tissue.

Thymus glands. In the thymus glands of litters from the high calcium carbonate diet-groups, the line of demarcation between cortex and medulla tended to be less distinct, which suggested that the organ had undergone premature involution (Figs. 10 and 11); but there were no signs of sclerosis or of degeneration in the thymocytes (Fig. 12). No differences were found in the thymus glands of dams.

Kidneys. The only abnormal features were early degenerative changes - cloudy swelling - in the tubule cells of a few of the most anaemic weanlings. There was no evidence of extensive fatty degeneration.

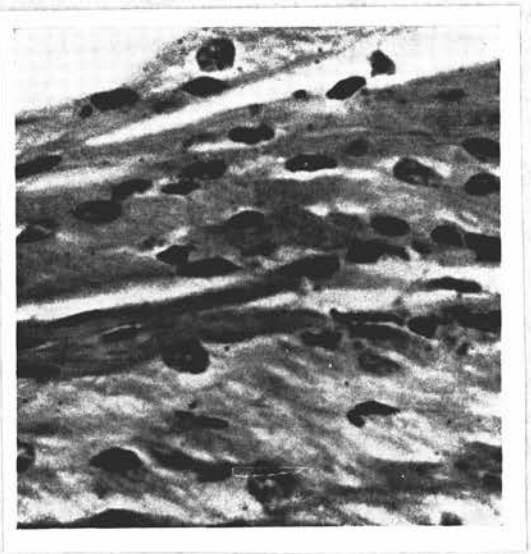
Spleens. There were no important differences in the structure of the spleens of either litters or mothers on different dietary treatments.

Livers. A certain amount of visible fat was present in sections of the livers of both adult and young mice on even the best diets in this experiment. In a few specimens the quantity of fat was small, but in many it was considerable, and in some it was enormous (Figs. 13-15).

In the weanlings, the impression was gained that the degree of fatty change was greatest when the level of calcium carbonate in the mother's diet was highest. In order to corroborate this, the amount of visible fat in comparable sections from a mother and a weanling in each diet-group was assessed by an arbitrary +, ++, +++ system. With only one observation from

FIGURE 9

Exp. 1. Heart Muscle



Early degenerative changes. Weanling from high calcium carbonate diet-group. (Haemalum and eosin; x 700)

FIGURES 10-12

Exp. 1. Thymus glands of weanlings

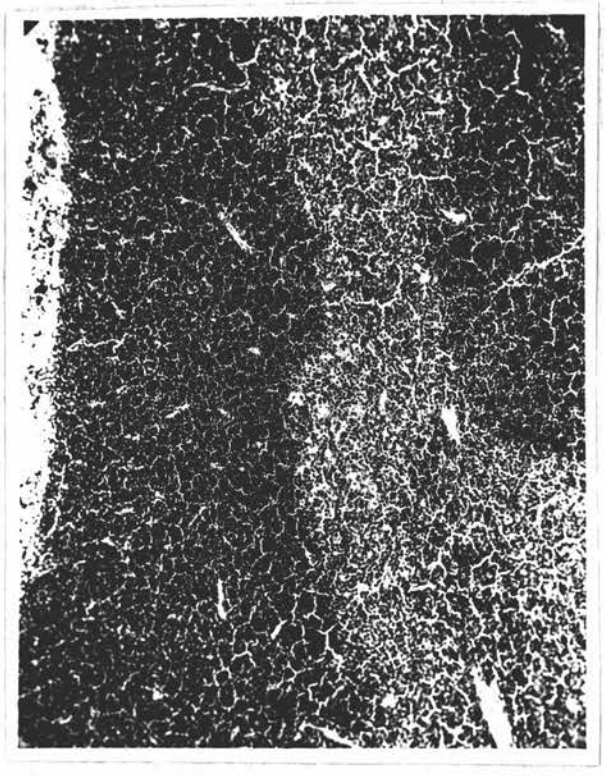


Fig. 10. Low calcium carbonate diet-group. (Haemalum and eosin; x 100)

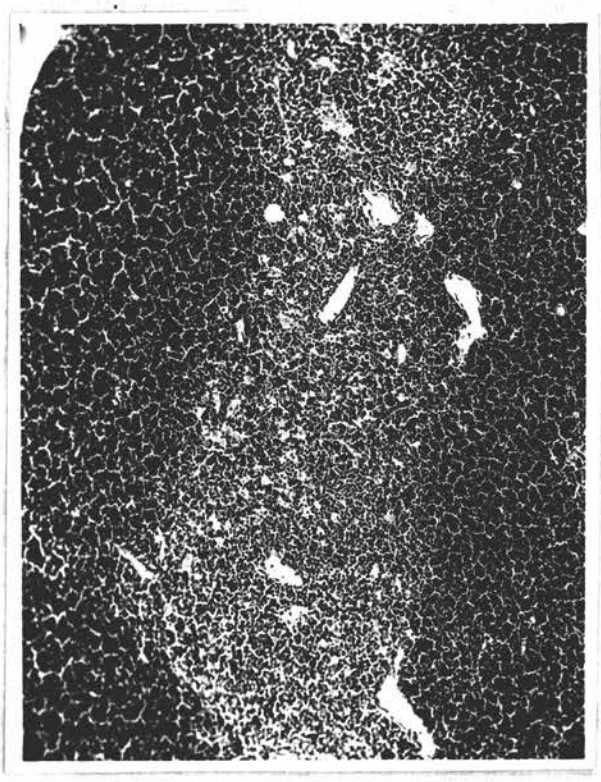


Fig. 11. High calcium carbonate diet-group. (Haemalum and eosin; x 100)

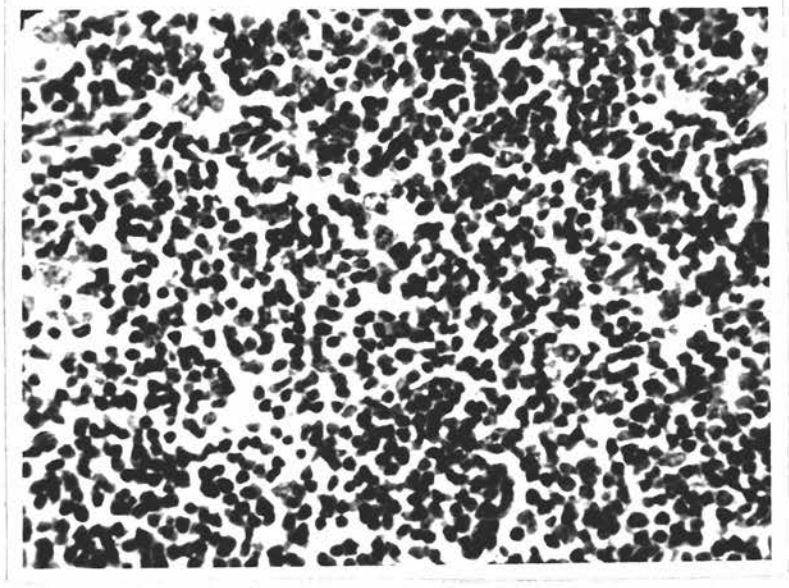


Fig. 12. Cortex. High calcium carbonate diet-group. (Haemalum and eosin; x 485)

FIGURES 13-16

Exp. 1. Livers of Weanlings

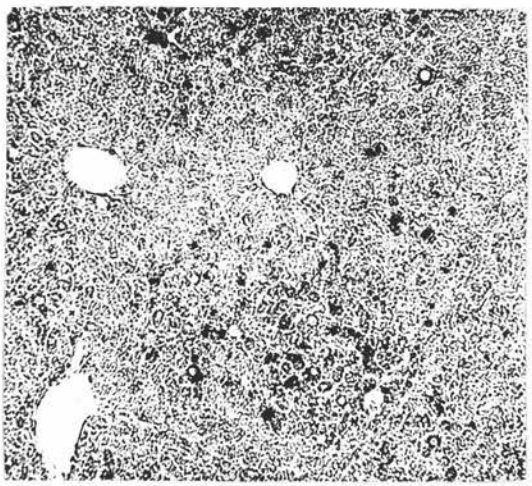


Fig. 13. Low calcium carbonate diet-group. (Scharlach R and haemalum; x 108)

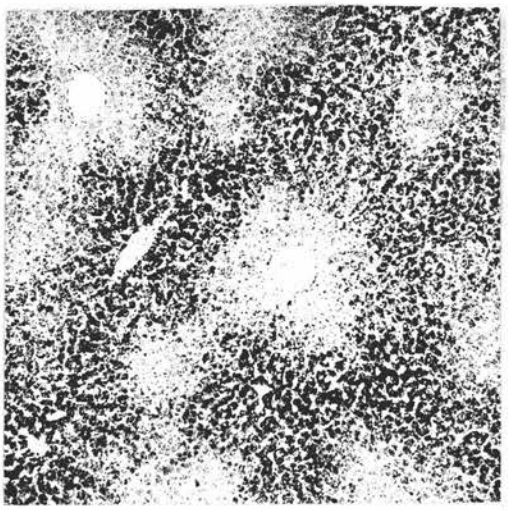


Fig. 14. Medium calcium carbonate diet-group (Scharlach R and haemalum; x 80)

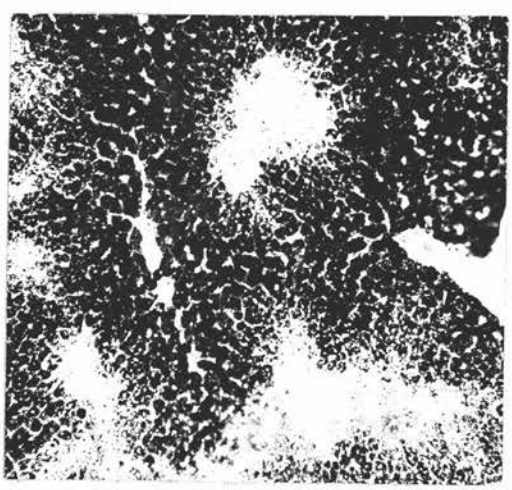


Fig. 15. High calcium carbonate diet-group. (Scharlach R and haemalum; x 95)

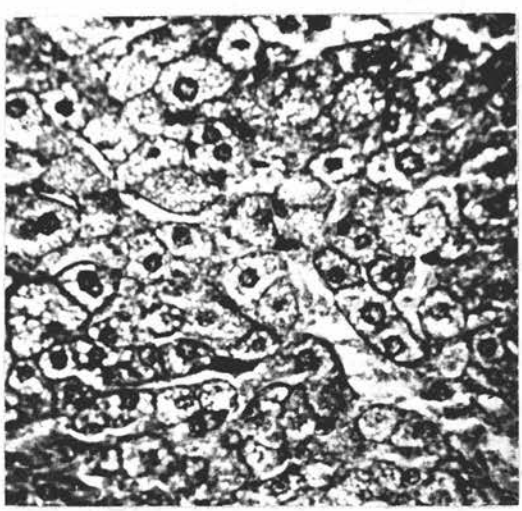


Fig. 16. High calcium carbonate diet-group (Haemalum and eosin; x 400)

each diet-group, the results were not suitable for statistical analysis. Nevertheless they confirmed the impression, since in the weanlings the highest number of '+'s was recorded from the diet-groups with the highest level of calcium carbonate, and the lowest number from the two groups with the lowest calcium carbonate levels* (Text Table 16). No association was apparent between the basal diet of each row and the amount of fat present in the liver.

The livers of the mothers were much more variable in their fat content, and no general correlation between liver fat and diet was evident (Text Table 17). It was nevertheless true that those livers with the least fat came from mice on the lower calcium carbonate diets, and those with the most from animals on the highest level of this dietary supplement.

The distribution of the fatty changes within the lobule was also studied. When the amount present was small, the fat was generally distributed fairly evenly throughout each lobule (Fig. 13). On the other hand, greater amounts of fat were almost invariably situated in the centrilobular areas (Fig. 14). In the most severe cases, nearly every parenchymatous cell was affected, the appearance being almost that of adipose tissue, relieved only by small patches around the portal vessels (Fig. 15).

The essential lesion in the hepatic cells was fatty degeneration. The earliest changes were manifested in the characteristic 'foam-cells' (Fig. 16), in which the cytoplasm was partly broken down and contained droplets of fat. In more advanced stages, the cells were swollen and contained large vacuoles; and in the most severe cases only the cell wall remained, sometimes with the remains of the nucleus close against it, the cytoplasm having been completely replaced by immense fat globules.

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*I am indebted to Mr. G. H. Cushnie, of the Pathology Department, Rowett Research Institute, for making a second, and independent, assessment of the sections.

TEXT TABLE 16.

Exp. 1. Litters. Histological estimates (from 0 to 4) of quantity of visible fat in frozen sections of liver prepared from weanlings representative of each treatment

(a) Observer A (W.A.G.)

	Col. 1	Col. 2	Col. 3	Col. 4	Mean	Stock diets
Row 1	2	1	3	2	2.00	
Row 2	1	3	1	3	2.00	
Row 3	2	0	2	2	1.50	
Row 4	1	1	2	3	1.75	
Mean	1.50	1.25	2.00	2.50	1.81	0.33

(b) Observer B (G.H.C.)

	Col. 1	Col. 2	Col. 3	Col. 4	Mean	Stock diets
Row 1	2	1	3	3	2.25	
Row 2	0	3	1	3	1.75	
Row 3	2	0	2	2	1.50	
Row 4	1	0	1	3	1.25	
Mean	1.25	1.00	1.75	2.75	1.69	0.33

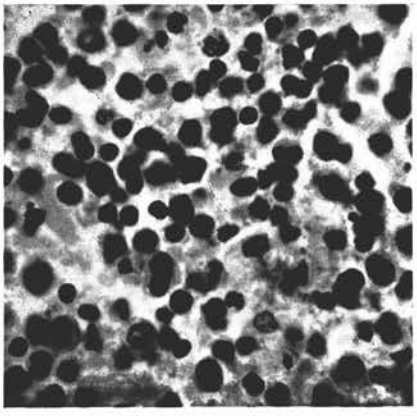
TEXT TABLE 17.

Exp. 1. Dams. Histological estimates (from 0 to 4) of quantity of visible fat in frozen sections of liver prepared from dams representative of each treatment.

	Col. 1	Col. 2	Col. 3	Col. 4	Mean	Stock diets
Row 1	4	3	2	2	2.75	
Row 2	2	3	2	2	2.25	
Row 3	3	3	4	2	3.00	
Row 4	2	3	3	4	3.00	
Mean	2.75	3.00	2.75	2.50	2.75	1.00

FIGURE 17

Exp. 1. Bone marrow



Weanling from high calcium carbonate diet-group. (Haemalum and eosin; x 650)

Bone marrow. All the bone-marrow specimens examined were highly cellular, with hyperplasia of the erythroid cell series. The reaction was most marked in marrows from the high calcium carbonate groups, which contained an abundance of immature erythroid cells with pyknotic nuclei and basophilic cytoplasm. The majority were early and intermediate normoblasts, only a few being in the later stages of development. All were normal or small in size, with no evidence of megaloblastosis (Fig. 17).

(iv) Other Observations

(a) Organ weights

It has already been mentioned (p. 5) that during the course of the experiment Dr. Marion Richards[*] began to make recordings of the weights of certain organs dissected from the experimental animals. These observations, when statistically analysed, showed that the addition of calcium carbonate to the mother's diet significantly increased the heart weights of the weanlings (expressed in mg. per 100 g. body-weight), but its effect on the dams' heart weights was not significant. The effects of the supplement on the weights of other organs also failed to reach significance, although - bearing in mind the incompleteness of the data - there was some indication that the weight of the thymus glands of the weanlings may have been reduced.

(b) Correlation between heart weight and blood haemoglobin concentration

The fact that both the relative heart weights (i.e. heart weights expressed as a percentage of body-weight) and the blood haemoglobin concentration (Hb) of weanlings were significantly affected by supplements in the dams' diets led me to examine the relationship between these two

[*] Dr. Richards was entirely responsible for these observations, but the main findings must be briefly mentioned here. A fuller description of the work is given by Richards and Greig (1952).

FIGURE 18

Exp. 1. Litters. Correlation between haemoglobin concentration and heart weight

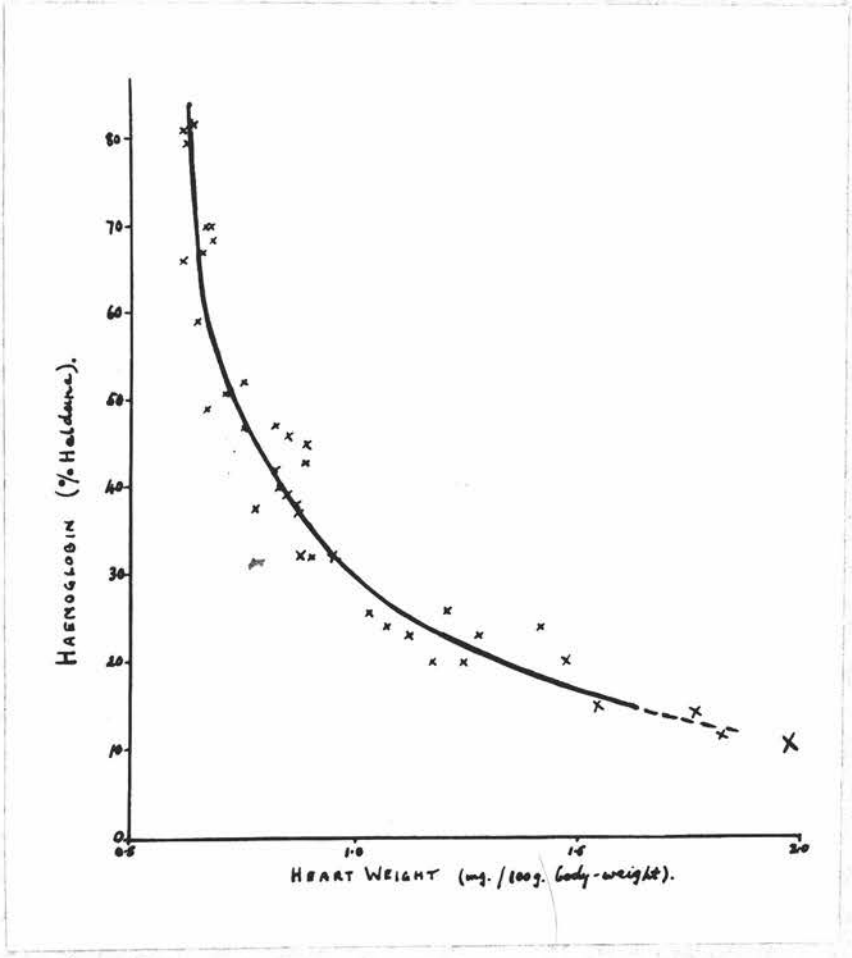
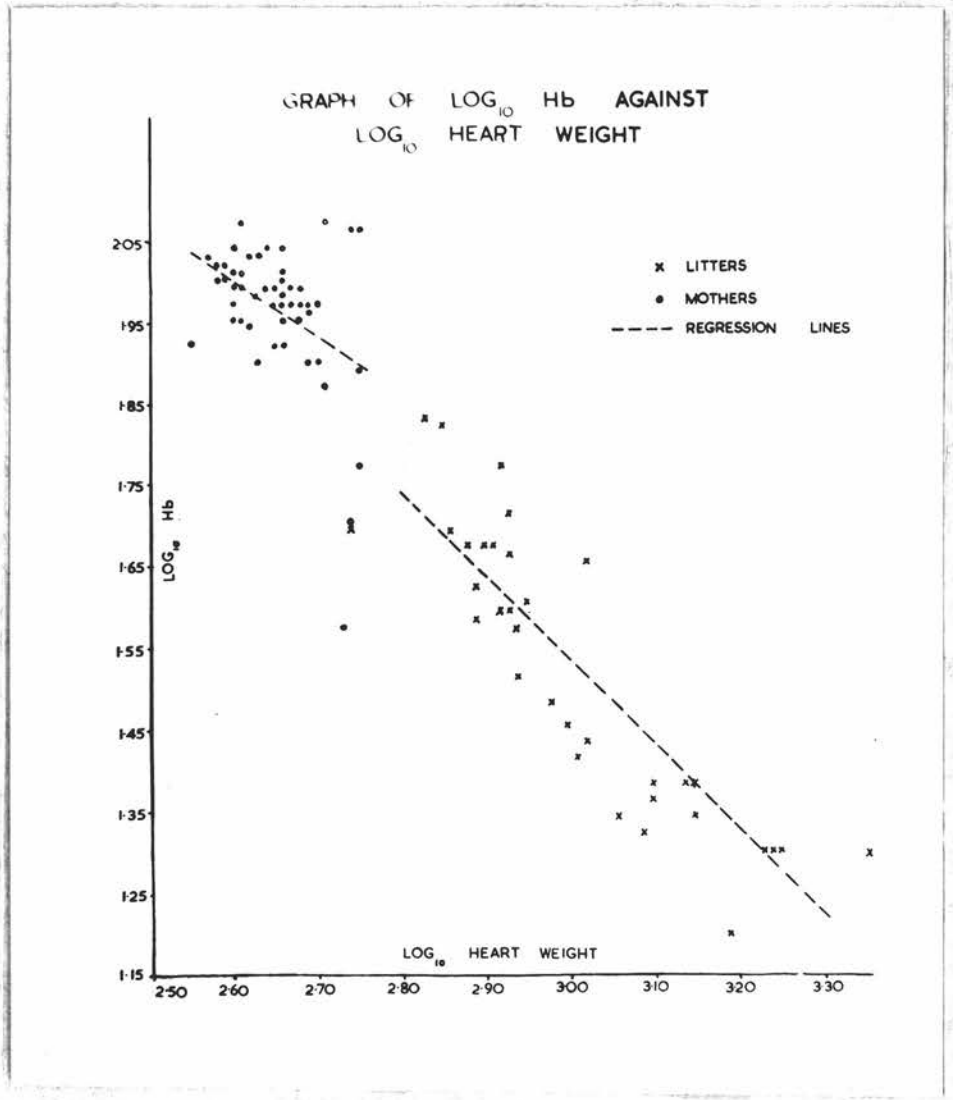


FIGURE 19

Exp. 1. Litters and Dams. Correlations between the logarithms of haemoglobin concentration and heart weight



attributes. When they were plotted against one another, ignoring treatment differences, a smooth curve resulted (Fig. 18), which could be transformed into a straight line by using the logarithm of each value instead of the value itself. This line is shown in Fig. 19, and was found (by Mr. M. H. Quenouille) to have the equation:

$$\log_{10} \text{Hb} = 4.610 + \underline{b} \log_{10} \text{H.W.\%},$$

$$\text{where } \underline{b} = -1.025 \pm 0.1111,$$

and where Hb is expressed as a percentage of the Haldane standard (100% = 14.8 g. haemoglobin/100 ml. blood), and H.W.% represents mg. heart-weight/100 g. body-weight.

When the logarithms of the observations on the dams were similarly treated, the following regression line was obtained:

$$\log_{10} \text{Hb} = 3.884 + \underline{b} \log_{10} \text{H.W.\%}$$

$$\text{where } \underline{b} = -0.724 \pm 0.2007,$$

and where Hb and H.W.% are expressed in the same terms as before (Fig. 19).

Mr. Quenouille also showed that the slopes of these two lines were not significantly different from each other, and from an analysis of the combined data for litters and dams, again ignoring treatment effects, he reported that the following line could be considered to represent the values for both series:

$$\log_{10} \text{Hb} = 5.061 + \underline{b} \log_{10} \text{H.W.\%},$$

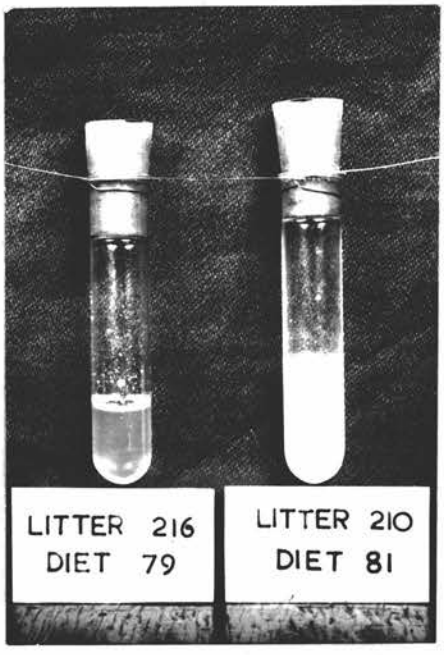
$$\text{where } \underline{b} = -1.171 \pm 0.0438,$$

and where Hb and H.W.% are expressed in the same terms as before.

Thus a negative correlation between Hb and H.W.% appears to have been established in both cases, although the variability was much less in the litters than in the dams.

FIGURE 20

Exp. 1. Litters. Blood Serum



(Left) low calcium carbonate diet-group.

(Right) high calcium carbonate diet-group.

(c) Plasma lipoids

It was noticed that when tubes containing samples of heparinised blood had been standing for some time in a rack, the plasma which separated to the top of the sample was frequently thick, opalescent and milky. This effect was most obvious in the most anaemic specimens of weanlings' blood. The general characters and appearance of the material suggested that it was fatty in nature, and tests of the plasma with osmic acid proved that this was so. Many of these instances of hyperlipaemia were extremely marked (see Fig. 20).

C. SUMMARY OF PART I

1. In an experiment conducted by Dr. Marion Richards it was shown that the addition of calcium carbonate at the rate of 2% to the diet of breeding mice seriously impaired breeding performance, especially rearing performance. The basic ration was a modification of the B diet of Sherman.

2. During the course of the experiment, I was invited to study the experimental animals from a pathological point of view. At weaning (21 days old), the progeny of dams with 2% of added calcium carbonate in their diet were ill-developed, malnourished and lackadaisical. At post-mortem examination they were found to be oedematous and usually overloaded with depot fat. Their blood was pale creamy pink in colour. Their hearts were enlarged, their thymus glands small and involuted, and their livers pale, friable, greasy and mottled. These findings suggested the existence of anaemia.

3. Haematological examination of these animals and their controls confirmed that calcium carbonate supplements to the maternal diet caused anaemia in the young at weaning; there was some evidence that it was hypochromic and

microcytic in type, and was accompanied by a normoblastic reaction in the bone marrow.

4. The dams on the high calcium carbonate diets were themselves anaemic, the anaemia being hypochromic in type and accompanied by leucopenia.

5. All the experimental dams on all the modified Sherman B diets, and also their progeny, were anaemic when compared with similar animals on stock diets. The anaemia in these cases appeared to be microcytic.

6. Histological examination of the organs of both weanlings and their dams showed that calcium carbonate supplements to the maternal diet resulted in hypertrophy of the cardiac muscle fibres, oedematous separation of the muscle bundles, and, in some weanlings, degenerative changes in the heart-muscle cells. The thymus glands of weanlings appeared to have undergone premature involution, and in some cases there were early degenerative changes in the cells of the renal tubules. The livers of these weanlings contained excessive quantities of fat.

7. There was a negative correlation between blood haemoglobin concentration on the one hand, and heart-weight relative to body-weight on the other; the correlation was most striking in the weanlings.

8. The most anaemic weanlings exhibited hyperlipaemia.

PART II

THE EFFECTS OF DIETARY CALCIUM CARBONATE AND ITS MODE OF ACTION

PART II.

A. INTRODUCTION

The studies described in Part I indicated that the addition of calcium carbonate to the diet of breeding mice could cause profound effects. The most obvious of these were an impairment in breeding performance, especially rearing performance, and the development of a severe anaemia in both mothers and young.

Such striking results were unexpected, since the calcium content (0.34% as Ca) of the original ration was by no means high, while its calcium : phosphorus ratio (Ca : P = 0.7 : 1) must be regarded as definitely low. Further, the amounts of calcium carbonate employed were quite moderate. The maximum rate of supplementation was 2%, making the total calcium (as Ca) content of the ration about 1.1%, and the calcium : phosphorus (Ca : P) ratio about 2.3 : 1 (i.e. CaO : P_2O_5 = 1.4 : 1). While these figures, especially the Ca : P ratio, must certainly be regarded as high, yet they do not constitute very gross deviations from the values often employed in stock diets for laboratory animals (the stock diet used with success in the Rowett Research Institute has a calcium (as Ca) content as high as 1.28%, with a Ca : P ratio of 1.3 : 1); and there is little doubt that the calcium content of farm animals' rations frequently exceeds such levels.

It seemed that these results, quite apart from their academic interest, might ultimately prove to be of practical importance in the feeding of livestock, since ground limestone is used extensively - and sometimes indiscriminately - as a calcium supplement to the rations of farm animals.

Calcium carbonate (as ground chalk and/or limestone flour) is a compulsory constituent of National Cattle Foods Nos. 1, 2 and 3, the Ministry of Food limits being 1½-2%, with an upper limit for total calcium (as $CaCO_3$) as high as 3½% (i.e. 1.4% as Ca); the corresponding upper limit for National Pig Foods Nos. 1 and 2 is 2¾% (1.1% as Ca). Moreover, although these levels are already fully as high as the highest level fed to the mice, breeders frequently administer some additional form of mineral supplement, and limestone, dicalcium phosphate or bone flour form the basis of most such commercially-prepared products. In such cases the total calcium intake undoubtedly must be exceedingly high.

Excessive feeding of limestone has often been criticised because of the likelihood of rendering the calcium : phosphorus ratio unbalanced, and at various times there have been reports that the feeding of large quantities of calcium carbonate or limestone has proved harmful to farm animals - for instance, it has been said to depress growth in swine and poultry (Parkhurst and McMurray, 1932; McGowan and Emslie, 1934; Dunlop, 1935). Excessive dietary calcium is well-known to have rachitogenic properties, owing to the induction of a relative deficiency of phosphorus, and has also been associated with a variety of functional diseases in farm animals, such as infertility in cattle (Hignett, 1949, 1950), nervous disorders in pigs (Reimers and Smuts, 1932), and, in poultry, lowered hatchability (Titus, Byerly, Ellis and Nestler, 1937) and reduced egg production and feed efficiency (Gutowska and Parkhurst, 1942). Although the induction of a deficiency of available phosphorus has sometimes been assumed, the modus operandi of high levels of dietary calcium has not always been satisfactorily explained, nor is it by any means certain that deficiency of phosphorus will lead to such a variety of disorders. Thus the whole problem of the effects of dietary calcium

appeared to be one deserving of fuller investigation.

The main question presenting itself was simply this: in what manner, and by what means, did the calcium carbonate supplements affect the reproductive and haemopoietic systems of the experimental mice? There is a vast and complex literature devoted to the metabolism of calcium, and especially its relation to phosphorus in bone formation and structure; but surprisingly little is known of its association, direct or indirect, with the reproductive and haemopoietic systems. It will be more convenient to review in detail such literature as does exist on these subjects at a later stage, but it may be said here that it is very conflicting. There have been claims that the feeding of supplementary calcium salts will improve reproductive performance, and others that it will impair it; similarly, the ingestion of additional calcium has been said to cause anaemia, but there is also much good evidence that it can cure or prevent it. In view of this, and also because the observations made in Experiment 1 had been far from complete, it seemed important first of all to confirm and extend the findings of Experiment 1 by means of a full-scale experiment designed expressly for the purpose, in which planned observations could be made throughout its course. At the same time, a start could be made to the problem of elucidating the mechanism of the action of dietary calcium carbonate.

For at least four reasons I considered it best to approach this problem by studying the anaemia; these reasons were: (1) anaemia was a very marked feature of the pathological syndrome; (2) it was easily measurable in exact terms; (3) of all the pathological features observed, the anaemia appeared most likely to be a primary rather than a secondary effect; (4) the pathological features of many forms of anaemia are well recognised, and their significance understood, so that study of the characters of the anaemia would be likely to

provide some clue to its pathogenesis.

I decided also to approach the problem from another angle. Since a calcium supplement had induced certain effects, it might be possible to prevent these by 'locking up' the calcium in such a way as to render it innocuous. One of the most obvious methods of doing this would be to provide additional inorganic phosphate. This procedure could also provide the answer to another question - were the effects due primarily to phosphorus deficiency or disturbance of the Ca : P ratio rather than to a simple excess of calcium carbonate?

1. ANAEMIA

As mentioned earlier, there was evidence in Experiment 1 that the anaemia should be attributed to an interference with blood formation rather than to abnormal destruction or loss of blood; that is to say, to a dyshaemopoietic rather than a haemolytic or haemorrhagic anaemia. This evidence comprised the absence of icterus or signs of haemorrhage, while the anisocytosis and poikilocytosis were also highly suggestive of a dyshaemopoietic origin (Dacie and White, 1949). Accordingly, I decided to approach the problem on this assumption, and to look for the likeliest cause or causes of the disorder. Before discussing this question, however, it will be useful briefly to review the main principles of our present understanding of normal erythropoiesis, the factors required to maintain it, and the ways in which it may become disordered.

Erythropoiesis

Much remains to be discovered of the process of erythropoiesis and the factors controlling it, but certain main points are generally accepted. Various aspects of the subject have been reviewed by, among others, Isaacs (1937), Dacie and White (1949), Parsons (1933),

Ferguson (1940), Cartwright (1947), Schultze (1940), Gordon and Charipper (1947), Daughaday, Williams and Daland (1948), as well as Wintrobe (1946) and Whitby and Britton (1950).

There seems little doubt that, in early embryonic life, primitive red blood cells are formed first in the yolk sac and later in other mesodermal sites (Gilmour, 1941). As the organs differentiate, the liver and spleen and to some extent the pelvis of the kidney become sites of erythropoiesis. At about the middle of foetal life the bone marrow begins to function as a blood forming organ, and at birth, or certainly very shortly after birth, it is normally the only site of red cell formation.

The study of haemopoiesis has been greatly confused by inconsistencies in nomenclature. Sometimes four or five names have been used by different workers to describe what is apparently the same type of cell, and names used by some authors to describe aberrant forms have been used by others for normal cells. A further difficulty has been the lack of agreement on the origin of the red cell series - a question that is still not settled. But whether the monophyletic or one of the polyphyletic theories be favoured, there is general agreement that the earliest differentiated form of red cell precursor is a large nucleated cell with a relatively small amount of non-granular, basophilic cytoplasm. It contains no haemoglobin, and has been variously called a pronormoblast, a proerythroblast, a megaloblast, etc. Many modern British authorities prefer the term 'pronormoblast' for this cell; they use the word 'normoblast', qualified where necessary, to cover the more mature forms of nucleated red cell, and describe all immature cells collectively as 'erythroblasts'. The whole process is known as 'normoblastic erythropoiesis' to distinguish it from abnormal forms of cell development such as 'megaloblastic erythropoiesis' seen in Addisonian pernicious anaemia of man, 'micronormoblastic erythropoiesis' seen in iron deficiency, and 'macronormoblastic erythropoiesis' seen in treated iron deficiency. This form of nomenclature will be adopted throughout this work.

Three stages of normoblast are usually described, early, intermediate and late, but separation of these types is quite artificial and arbitrary. The essential changes seen in maturation are loss of nucleoli followed by a progressive shrinkage in the size of the cell. At the same time, the nucleus becomes relatively smaller and more pyknotic and the cytoplasm assumes acidophilic staining properties as the result of the formation of globin and haemoglobin. Finally the nucleus is lost as the ripening cell loses its spheroidal outline and becomes disc-like in shape, forming a reticulocyte. Reticulocytes - cells at the penultimate stage of maturation - react supravitally to certain basic dyes, and are probably always slightly larger than adult erythrocytes; certainly they are so when blood regeneration is active (Dameshek and Schwartz, 1940). The final stage is the mature, circulating erythrocyte.

The factors which govern the growth and differentiation of tissue cells remain obscure but there can be little doubt that two forms of nucleic acid - ribonucleic and desoxyribonucleic - play an important part in the processes. They are present in both the nuclei and cytoplasm

of all young, actively growing cells (Caspersson and Schultz, 1939), and are necessary for the synthesis of protein. Thorell (1947) studied developing erythroblasts, and showed that the primitive pronormoblasts contain the greatest amounts of ribose polynucleotides, which diminish in amount as the cell matures until, at the orthochromatic stage, none remains. During this period of maturation, basic cellular proteins such as globins are synthesised, and, later, haemoglobin is formed.

Haemoglobin, one of the group of respiratory pigments, is a protohemeglobin compound of large molecular weight (approximately 67,000), and can be considered to consist of globin, protoporphyrin IX and iron (Schultze, 1940). It is thought to be built up by the polymerisation of four haemochromogen molecules, each of which is itself a conjugated protein consisting of the protein globin and the prosthetic group haem (Anson and Mirsky, 1925). Haem, sometimes described as 'ferriprotoporphyrin' (Shack and Clark, 1947), is a compound of protoporphyrin IX (a group of four substituted pyrrol nuclei) with ferrous iron. It is both interesting and of some importance to note that although the haemoglobins found in various animal species are not identical (Schenck, 1930; Mirsky and Anson, 1932) and although there are also differences between the haemoglobins of adult and of foetal blood (Schenck, 1930; Brinkman, Wilderschut and Wittermans, 1934; Darling, Smith, Asmussen and Cohen, 1941), yet the differences lie solely in the amino-acid composition of the protein portion, the haem portion being identical in every case (Block, 1934; Beach, Bernstein, Himmel, Williams and Macy, 1939). This same haem prosthetic group is also found in a number of other respiratory pigments, such as catalase and the cytochromes (Anson and Mirsky, 1925; Stern, 1935-6; Granick and Gilder, 1947; Drabkin, 1951). However, certain other porphyrins have been described in growing cells (see Granick and Gilder, 1947), and recently Schwartz and Wikoff (1952) have described a coproporphyrin in reticulocytes; but at least some of these may be by-products of protoporphyrin IX synthesis (Rimington, 1938).

The exact mechanism of haemoglobin synthesis is not fully understood. It seems to be formed in situ in the erythrogenic tissue of the bone marrow, and according to Witts (1936) and others the final synthesis takes place within the nucleus of the developing cell. Certainly immature red cells contain iron in their stroma, and synthesise it to haem; they can absorb iron from their environment by means of acceptors in the stroma (Walsh, Thomas, Chow, Fluharty and Finch, 1949). Hawkins, Robscheit-Robbins and Whipple (1938) believed that globin is formed in the liver, and it is probable that the porphyrin fraction is synthesised in the marrow cells. Iron is available from the blood plasma, where it is held combined with a globulin (Schade and Caroline, 1946; Cohn, 1948).

It is not clear at what stage iron enters into the molecule. Schultze (1940) quotes evidence to suggest either that protoporphyrin combines with iron to form protohaematin which, after reduction to protohaem, combines with globin to form haemoglobin; or that a globin-protoporphyrin compound is first formed which yields haemoglobin upon the incorporation of iron. The process described by Vaughan (1948) is similar to the first of these alternatives.

It seems certain, however, that the globin portion is formed early, when the concentration of ribose polynucleotides in the cell is high, while

the haem fraction is formed only at a much later stage (Shemin and Rittenburg, 1946). Thorell (1947) was unable to detect porphyrin substances in developing red cells until they had reached the polychromatic stage, after which the concentration of haemoglobin rose rapidly. It would thus seem that polychromasia is not by itself a sign that haemoglobin synthesis has commenced, but may merely indicate the formation of acidophilic globin and the disappearance of the basophilic nucleotides. However, Israëls (1941) has shown that early haemoglobinisation of red cell precursors, which is a feature of megaloblastic erythropoiesis, is also seen when unusually sustained or sudden demands are made on erythrocyte production.

The average life of the red cell (at least in the rat and the human being) is now believed to be about 100-120 days (Berlin, Huff and Henessy, 1951; Callender, Powell and Witts, 1945; Shemin and Rittenburg, 1946; see also Ashby, 1948), after which it is broken up or lysed, the debris being removed by the reticulo-endothelial system. The breakdown process is not fully understood, but it is known that the protein is split up into its constituent amino-acids which are then added to the metabolic pool. From the broken-down haemoglobin molecules, iron is conserved for future use, while from the remaining portion, the pyrrholic compound bilirubin is formed and is excreted by the liver, but may be re-absorbed from the intestine.

Regulation of erythropoiesis

The regulation of erythropoietic activity is imperfectly understood, but there is much strong evidence to suggest that relative anoxia and a lowered oxygen tension in the bone marrow may constitute a trigger mechanism leading to increased activity. It is well-known, for instance, that increased erythropoiesis regularly follows blood loss, and it has been assumed that this is the result of a reduction in the oxygen supply to the marrow. Seventy-five years ago, Bert (1878) reported that Jourdanet had empirically recommended the use of rarefied air in the treatment of anaemia; Viault (1891) also maintained that the deficient atmospheric oxygen in mountainous regions was a strong stimulus for increased production of red cells and haemoglobin. That a secondary polycythaemia followed exposure to low oxygen and barometric pressure was shown by Campbell (1926-7, 1927), who also observed diminished red cell production in many species under the influence of a high oxygen tension. From a review of all the evidence, Sabin (1928) supported Campbell's conclusions, which have later been confirmed by several workers, notably Hurtado (1932), and Reinhard, Moore, Dubach and Wade (1944).

After a very full consideration of the literature, Hurtado, Merino and Delgado (1945) concluded that, in most cases at least, low barometric pressures induce a polycythaemia which is directly proportional to the degree, duration and continuity of the anoxic stimulus. They considered that this is the result, in the first place, of release of reserve stores of blood (see Barcroft and Barcroft, 1923-4), and, in the second place, of increased erythropoietic activity. On the other hand, leucopoiesis does not seem to be increased. They also concluded that a return to sea-level is followed by a reduction in erythropoiesis.

On the other hand, when rabbits were exposed to high oxygen concentrations, Karsner (1916) could find no pathological changes in the blood, spleen or bone marrow. Boycott and Oakley (1933) found not only that high oxygen concentrations failed to inhibit the regeneration of red blood cells after haemorrhage, but that the reticulocyte response was actually greater in an atmosphere containing 65% of oxygen. Grant and Root (1947a, b) noted that increased erythropoiesis continued for three weeks after a single large haemorrhage in dogs, although the oxygen saturation and tension of the blood had returned to normal after only three to five hours; while when several small haemorrhages were induced over a period of days, so that the oxygen tension did not fall, blood regneration still took place. Grant (1948a, b) concluded from an extension of this work, in which he examined the bone marrow blood directly, that vigorous erythropoiesis is not necessarily associated with a lowered oxygen saturation of the blood circulating through the marrow. Again, Rosin and Rachmilewitz (1948), working with explanted marrow fragments, found that erythroblastic cells, at low levels of atmospheric oxygen, showed degeneration and reduced mitotic activity. With 15% oxygen, growth was similar to that of control fragments in air, while with 50% oxygen there was an increased rate of maturation and multiplication. They pointed out that there is much evidence of reduced growth and activity with other tissue cells, such as fibroblasts, at low oxygen tensions, and brought forward the ingenious theory that the stimulation of marrow activity at high altitudes may be due, not to oxygen want, but to over-compensation initiated by the low oxygen tension of the atmosphere, such as increased respiration, increased arterial pressure, and increased cardiac rate and output. Thus there may in fact result a compensatory hyperoxaemia in the marrow vessels - "anoxic hyperoxia". In view of this suggestion, it is interesting to note that Hurtado et al. (1945) found evidence that when the lowering of atmospheric oxygen is very severe, decreased rather than increased erythropoiesis supervenes.

The situation is therefore complex, but it seems to have been established that a change in oxygen tension is one factor which can stimulate erythropoietic activity. The manner in which it acts, however, is far from clear. Davis (1940, 1941) has suggested that vasomotor control of the marrow vessels may exert some subsidiary effect on erythropoietic function. He found that the administration of drugs such as acetylcholine, which produce vasodilation in the marrow vessels, caused a reduction in the level of artificially-induced polycythaemias, while polycythaemia could be induced in normal animals with drugs such as ephedrine, which have the opposite effect on the marrow vessels. However, Warren (1941-2) showed that for erythroid hyperplasia to follow exposure to lowered atmospheric pressure, intact innervation to the marrow is not necessary. The same author, working on the hypothesis that the existence of an alteration in oxygen tension might be detected by chemoreceptors in the nervous system and the message then relayed to the marrow by humoral mechanisms, was unable to find any change in the marrow activity of normal animals after the injection of serum from an animal which had been subjected to low atmospheric oxygen. The pituitary gland may be involved, for Stewart, Greep and Meyer (1935) and Meyer, Stewart, Thewlis and Rusch (1937) have reported that the hypophysectomised rat does not respond to decreased oxygen tension with hyperplasia of the marrow. A more positive proposal has come from Petri and his co-workers

(Benkö, Petri, Eisner, Kardos, Szabó, Bentzik and Heténi, 1950; Petri and Bentzik, 1950; Petri, Benkö, Kardos, Eisner, Szabó, Bentzik and Heténi, 1950). They postulated that there is an interaction between the central nervous system and the bone marrow, the original effect being produced by local hypoxia in the marrow. When they induced hypoxia in the femoral bone marrow of dogs, either by ligation of the nutrient artery or by injection into it of potassium cyanide, erythrocytosis always followed unless the efferent medullary veins and their collaterals were ligated, or unless the spinal cord had been previously severed in the dorsal region. That the effect of ligation on the marrow was not merely local was also demonstrated by the fact that ligation of the nutrient artery of one limb nevertheless increased the erythrocytosis in both medullary veins.

Among the other factors which have a direct effect on the regulation of erythropoiesis is the haemopoietic principle of Castle, which may or may not be identical with vitamin B_{12}. This factor acts at the pronormoblast stage, and its absence affects the stroma, producing disordered erythropoiesis characterised by megaloblastosis (as in pernicious anaemia of man). The element cobalt is a constituent of vitamin B_{12}, but the cobalt ion itself appears to have a stimulating action on erythropoiesis, probably aiding in the release of cells from the marrow by some interference with the respiration of erythroblasts (Barron and Barron, 1936-7). Iron, besides having vital importance as a constituent of the haem group, has also been credited with a stimulating action at some other stage of erythropoiesis (Whipple and Robsheit-Robbins, 1930c; Fowler and Barer, 1941), possibly the maturation of reticulocytes (Josephs, 1931, 1939b; Fitzhugh, Robson and Drabkin, 1933; Haden, 1937). This view is not shared by Witts (1933).

Other regulating factors include the reticulocyte-ripening substances of Plum and his colleagues (Plum, 1942, 1943, 1944a, b, 1946, 1947a, b, c; Jacobsen and Plum, 1943; Christensen and Plum, 1947; Clemmesen, Espersen and Plum, 1948). They found that certain factors necessary for the ripening of reticulocytes are present in liver extracts and also in normal serum. A thermolabile factor found in high concentration in the stomach mucosa can be activated by tyrosine to form the factor found in the serum; the hypophysis and thyroid glands also play a part in its activation. In every form of anaemia examined there was an increased concentration of this substance in the serum, and the plasma of individuals who had been exsanguinated was more stimulating to the growth of marrow explants than was normal serum. Hays (1946) has described another factor in highly purified liver extracts which assists the maturation of normoblasts into reticulocytes. He also studied the effects of folic acid (vide infra) on the maturation of primitive red cells, and concluded that it does not act on them directly. Norris and Majnarich (1948), however, have identified xanthopterin as a stimulant to marrow proliferation.

It also seems to be established that erythropoiesis is to some extent influenced by certain endocrine secretions, and among the glands for which claims have been made are the pituitary, thyroid, gonads, adrenals and pancreas. Attempts to isolate a specific haemopoietic hormone have proved generally unsuccessful, although the existence of such an entity has been postulated by Moehlig and Bates (1933) and Evans (1935). Flaks, Himmel

and Zlotnik (1937, 1938) have claimed that a thermostable factor in the "fraction desalbuminée" of the anterior lobe of the pituitary gland will stimulate erythropoiesis in intact, hypophysectomised or thyroidectomised rats, and Dutch workers (Overbeek, 1936; Querido and Overbeek, 1938, 1939; Overbeek and Querido, 1938) have made similar claims; but none of them seem ever to have been confirmed.

It seems certain, however, that the hormones of the pituitary gland bear some relation to the blood picture. Anaemia has regularly been observed after hypophysectomy (Aschner, 1912; Houssay, Royer and Orias, 1931; Stewart, Greep and Meyer, 1935; Meyer, Stewart, Thewlis and Rusch, 1937; Vollmer, Gordon, Levenstein and Charipper, 1939; Crafts, 1941a, b; Christensen and Plum, 1947), while a number of other experimental and clinical reports also suggest that the pituitary gland is concerned, directly or indirectly, with erythropoiesis (Schulhof and Matthies, 1927; Silver, 1933; Snapper, Groen, Hunter and Witts, 1937). Although macrocytic anaemia has been recorded (Snapper et al., 1937; Witts, 1942), the anaemia of pituitary deficiency is usually microcytic and hypochromic (Silver, 1933; Crafts, 1946a, b), and is associated with hypoplasia of the erythroid elements of the bone marrow (Vollmer et al., 1939; Crafts, 1946a, b). It has been suggested that these effects of pituitary insufficiency may be associated with effects on various endocrine organs (Vollmer and Gordon, 1941), such as the thyroid gland (Crafts, 1946b; Meyer, Thewlis and Rusch, 1940), the adrenal gland (Moehlig and Bates, 1933; Dougherty and White, 1944; White and Dougherty, 1945), the gonads (Crafts, 1946a; Watkinson, McMenemy and Evans, 1947); also on iron and copper metabolism (Crafts, 1946b), gastric acidity (Snapper et al., 1937; Crafts and Walker, 1947a), and even availability of essential proteins through interference with appetite (Crafts and Walker, 1947b; Wintrobe, 1946). There is evidence that neither gonadotrophic hormone (Moffat, 1940) nor prolactin (Querido and Overbeek, 1939) are involved, but Davis (1941, 1942) found that injections of posterior pituitary extract increased the blood haemoglobin concentration and the red cell count (even in splenectomised animals, thereby excluding the possibility of simple release of stored blood by means of vasoconstriction). However, the work of Gilman and Goodman (1935-6), Dodds and his co-workers (Dodds and Noble, 1935; Dodds, Hills, Noble and Williams, 1935) and also McFarlane and McPhail (1937) contradicts this, since they found that injections of posterior pituitary extract resulted in anaemia.

The hormones of the adrenal glands also appear to be capable of affecting the formed elements of the peripheral blood, although, in contrast to the hypophyseal hormones, the leucocytic picture is usually more affected than the erythrocytic (Zwemer and Lyons, 1928; Reinhart and Holmes, 1940; Majumder and Wintrobe, 1948). Addison, in 1885, described anaemia as one of the symptoms accompanying disease of the adrenal cortex, and Corey and Britton (1932) found that adrenalectomy increased the red cell count in cats by 50-100%, but attributed this effect to loss of fluid. Haemoconcentration following this operation in dogs and cats has also been reported by Stewart (1926), Rogoff and Stewart (1926) and Britton and Silvette (1931). On the other hand, Simpson, Dennison and Korenchevsky (1934) noted slight anaemia following adrenalectomy in rats and White and Dougherty (1945) and Hill (cited by Collip, 1935) observed that repeated

daily injections of adrenotrophic hormone caused increase in blood haemoglobin and reticulocytosis. Vollmer, Gordon and Charipper (1942) found that desoxycorticosterone acetate, or cortical hormone, did not repair the marrow hypoplasia of hypophysectomy, although it did maintain the red cell levels for some time. Many investigators including Nice, Morris and Elhardt (1930), Lucia, Aggeler, Husser and Leonard (1937), and Davis (1942) have reported that injections of epinephrine also cause polythaemia, but most of these workers suggest that the site of action of this hormone is probably not the erythropoietic tissues.

That the internal secretions of the gonads affect the blood picture is well recognised, and it is universally agreed that both the blood haemoglobin concentration and the number of red cells are higher in normal males than in normal females in a wide variety of species (see review by Daughaday, Williams and Daland, 1948). Androgens stimulate erythropoiesis and oestrogens depress it (Steinglass, Gordon and Charipper, 1941; Vollmer and Gordon, 1941; Crafts, 1941b; Finkelstein, Gordon and Charipper, 1944; Stein and Jacobsen, 1944; Taber, Davis and Domm, 1942-3; Glass, 1943; Domm, Taber and Davis, 1943). McCullagh and Jones (1942) reported that testosterone, which raised the low haemoglobin levels of eunuchoid men, also raised their basal metabolic rate, and concluded that erythropoiesis and B.M.R. might be related phenomena. The modus operandi of androgens on the blood picture is not clear - they do not simply antagonise oestrogens (Tyslowitz and Dingemanse, 1941). Oestrogens, however, appear to cause an anaemia associated with a definite marrow hypoplasia (Castrodale, Bierbaum, Hellwig and Macbryde, 1941; Tyslowitz and Dingemanse, 1941; Vollmer and Gordon, 1941; Taber, Davis and Domm, 1942-3; Crafts, 1948). Newcomer (1947) studied the influence of several organs on the blood of pregnant rats, and concluded that neither the ovary nor the foetus had any direct effect; the presence of an active placenta, however, was strongly anaemigenic, although the anaemia was possibly not hormonal in origin but simply an effect of hydraemia.

There is general agreement that the process of erythropoiesis is influenced by the thyroid gland, though it is not clear whether thyroxin exerts a direct effect on the bone marrow. It may be that marrow activity is related simply to metabolic rate, or, on the other hand, the effect of thyroid hormone may be mediated through one or more of the other endocrine organs. The whole question of the influence of the thyroid gland on erythropoiesis will be reviewed in detail at a later stage.

Factors required for erythropoiesis, and the general causes of dyshaemopoiesis

In the healthy adult, erythropoiesis proceeds at such a rate as just to compensate for the ordinary wastage of corpuscles. But it is a dynamic process and in disease it may become accelerated, slowed or deranged. Dyshaemopoiesis can result from a deficiency of the factors essential for erythropoiesis or from a failure to utilise these factors properly, and it also follows disorders of the mechanism responsible for the regulation of haemopoietic activity.

The factors necessary for normal red cell formation may be divided

into three classes - those which contribute to the stroma material, those which contribute to the haemoglobin molecules, and those which act either as catalysts to the processes of synthesis or as general stimulants and activators of normoblastic erythropoiesis. Many of them will require detailed consideration at a later stage of this work, but for the present a brief summary of the main points is presented.

According to Erickson, Williams, Bernstein, Arvin, Jones and Macy (1938) and Beach, Erickson, Bernstein, Williams and Macy (1939), the red cell stroma consists largely of protein and lipoids, existing, apparently, in the form of a lipid-protein complex. Erickson et al. (1938) have shown that in at least five mammalian species, the lipoids (which constitute 20-25% of the stromal material) consist largely of phospholipid (60%), cholesterol (30%) and cholesterol esters and neutral fat (10%). About half of the phospholipid is cephalin and the remainder lecithin and sphingomyelin. The composition of the protein fraction varies with the species. However, Beach et al. (1939) have shown that, in five mammalian species, certain amino-acids were present in practically identical ratios, as follows: histidine 7, arginine 14.5, lysine 13, tyrosine 8, tryptophane 3, cystine 2 and methionine 3.5.

Some of the materials necessary for haemoglobin building have already been indicated. The formation of the haem prosthetic group requires the synthesis of porphyrin rings from pyrrols; the mechanism of the synthesis is unknown, but it is believed that at the starting point comparatively simple molecules such as glycine are utilised (Shemin and Rittenburg, 1946; London, Shemin and Rittenburg, 1948), together with acetic acid, aceto-acetic acid, proline, hydroxyproline and tryptophane (Whitby and Britton, 1950). There is, however, some evidence that animals can make use of pre-formed pyrrhols, such as chlorophyll, for haemoglobin formation (Minot, 1934; Patek, 1936; Kirkman, 1939). An adequate supply of non-haematin iron is obviously also essential to haem formation.

The globin portion of haemoglobin is said to consist of at least fifteen amino-acids (Schmidt, 1938). Whipple and Robscheit-Robbins (1930b) found that various meats and offals, and also gelatin, assisted haemoglobin regeneration, and the same authors (1940) and Robscheit-Robbins (1947) list the following amino-acids as being probable constituents of the globin protein: histidine, phenylalanine, proline, cystine, glutamic and aspartic acids, glycine, valine, iso-leucine, arginine, alanine, leucine, methionine, lysine, tryptophane and tyrosine. They found that isomeric and dl-synthetic forms were as effectively utilised as natural forms. Threonine and serine have also been isolated from some globins (Block and Bolling, 1943).

Among the substances which act as catalysts to erythrocyte production is copper (Hart, Steenbock, Waddell and Elvehjem, 1928; Keil and Nelson, 1930-31; Elvehjem and Sherman, 1937). Copper is not a constituent of the haemoglobin molecule, although it is contained in the stroma of the red cell (Elvehjem, Steenbock and Hart, 1929a; Tompsett, 1934). Traces of copper are essential for the incorporation of inorganic iron into haem, acting possibly by catalysing the formation of a ferro-porphyrin (Cunningham, 1931) or by assisting in the mobilisation of iron from the storage depots (Hutchison, 1938). Some workers (Stein and Lewis, 1933; Wickwire, Burge

and Krouse, 1936; Smith and Medlicott, 1944) also believe that copper assists erythropoiesis in other ways, such as stimulation of the maturation and release of red cells from the marrow. A number of other elements - such as manganese, cobalt, nickel, zinc, chromium, selenium, vanadium, germanium, titanium, mercury, molybdenum and arsenic - have been the subjects of similar claims, but these have not been generally substantiated.

A number of vitamins also have a direct or indirect effect on erythropoiesis, notably vitamin C and certain members of the vitamin B complex. Among these are folic acid, riboflavin, nicotinic acid, choline and pyridoxin, and an inadequate supply of any of these substances might therefore result in dyshaemopoiesis. Under certain other conditions, such as sepsis or cachexia, the body is unable to utilise the blood-building materials, while certain chemical poisons, such as lead, will also interfere with normal red cell production. Direct damage to the bone marrow, by such means as benzol, X-rays or radium, will also result in dyshaemopoiesis.

Possible causes of dyshaemopoiesis in Experiment 1

In considering in what respect erythropoiesis had become disordered as a result of the dietary supplements of calcium carbonate fed to the mice in Experiment 1, certain of the above causes of dyshaemopoiesis could be immediately ruled out. For instance, there had been no exposure to changes in atmosphere oxygen tensions, nor to X-rays or radium. There had been no sign of sepsis. It was most unlikely that chemical poisons had been introduced, as - quite apart from the hyperplastic state of the bone marrow - the calcium carbonate used had been an Analar brand, with a confidence limit for heavy metals of 0.002%. Finally, while it is true that cachexia had been a feature of many of the anaemic animals, there had been no obvious cause for this apart from the anaemia itself; and so it appeared that cachexia had followed anaemia, not vice versa.

The best clues were offered by the condition of the bone marrow and the peripheral erythrocytic picture. The generalised hyperplasia of the marrow eliminated from consideration of all causes which would lead to an aplastic anaemia, and indicated that the marrow was trying to respond to the body's call for more

circulating haemoglobin. However, interference in the release of ripe cells from the marrow was clearly not the major cause of the disorder. Not only had the peripheral blood contained numerous immature red cells, but its low M.C.H. had indicated that the number of circulating red corpuscles had been higher than would have been expected from the haemoglobin concentration. In addition, the great majority of the erythroblasts in the bone marrow had been early or intermediate normoblasts, not more mature cells. There must therefore have been some interference in the formation either of haemoglobin or of the red cell stroma.

From what has been said in regard to the sequence of the steps in erythropoiesis, it follows that, when stroma formation is inefficient, normal development of the erythroblast must come to an end at about the pronormoblast stage. The marrow is unable to produce the number of erythrocytes demanded of it, and its reaction to the call for more circulating haemoglobin is to produce large cells carrying as much pigment as possible; consequently, any cells finally released and also cells in the course of development are larger (though variable in size) and contain more haemoglobin than normal cells, while the proportion of primitive cells in the marrow is increased. This situation - which is, of course, well recognised in such conditions as pernicious anaemia in man - is known as hyperchromic macrocytic anaemia with megaloblastic erythropoiesis. In this form of anaemia, therefore, the M.C.H. and M.C.V. are high, and the M.C.H.C. is not reduced. Clearly it bears no resemblance to the picture presented in the experimental mice, where, as has been seen, the anaemia was certainly neither hyperchromic nor macrocytic, and indeed was probably hypochromic and possibly microcytic. By this reasoning, therefore, the chief defect in erythropoiesis must have lain in the synthesis of haemoglobin rather than in the formation of the cell stroma.

When haemoglobin synthesis is defective, the cell stroma nevertheless grows under the influence of ribose polynucleotides until it is largely complete. This occurs at the early normoblast stage, and thereafter no further proper differentiation can take place. Consequently the cell remains relatively basophilic, although, if the formation of globin has not been interfered with, some degree of polychromasia will be evident; and unless haemoglobin synthesis is completely inhibited a proportion of cells will mature to the late normoblast and reticulocyte stages.

As the result of an inability to synthesise haemoglobin, unpigmented normoblasts pile up behind the bottleneck, and at the same time the resultant anaemia leads to anoxia; this then stimulates the marrow to produce increased numbers of erythroblastic precursors, which in turn reach the normoblast stage but can proceed no further.* However, unless the impediment to haemoglobin synthesis is very severe, a number of cells differentiate sufficiently to obtain their release into the circulation; it may be that the congestion of cells in the marrow serves to force some immature cells out of their developmental sites. These cells do not contain their full quota of haemoglobin, however, and are consequently hypochromic, while the smaller cytoplasmic mass necessary to carry a reduced amount of haemoglobin results in their becoming microcytic. The incompletely haemoglobinised normoblasts in the bone marrow are also abnormally small in size - a condition known as micronormoblastic erythropoiesis. Clearly, the longer the anaemia persists the greater will be the proportion of anisocytosis, and after 120 days

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* In iron-deficiency anaemia, Dacie and White (1949) have suggested that not only is there inadequate synthesis of haem, but the low level of plasma iron - which is a feature of this condition (Moore, Doan and Arrowsmith, 1937) - may result in a failure of the last stages of normoblast differentiation; and if it is true that copper also plays a part in the final maturation and release of erythroblasts (see p. 132), then a similar situation could arise in copper deficiency.

(the average life of an adult erythrocyte) almost all the circulating red cells will be microcytic.

A gross hyperplasia of the erythroid elements in the bone marrow may also be reflected in a reduction in the number of circulating myeloid cells. This state of affairs is presumably due simply to the fact that in the congested marrow there is insufficient space for the normal number of myeloblasts to develop; while, if the monophyletic theory of haemopoiesis be accepted, it could also be argued that the strong demand for erythrocytes diverted a proportion of potential granulocytes into erythroblastic differentiation.

The tentative findings obtained from Experiment 1 agree very well with the above description of the principal blood and bone marrow changes seen in dyshaemopoiesis associated with a failure to synthesise haemoglobin. They therefore suggest that such a failure must have been at least the chief cause of the disorder. In considering its possible causes, it may be said at once that an insufficiency of the globin moiety was highly unlikely to be one of them. Anaemia due to deficiency of protein is rare, but has been produced experimentally in animals (Kyer and Bethell, 1938; Hahn and Whipple, 1939; Whipple and Robscheit-Robbins, 1940; Orten and Orten, 1945; Robscheit-Robbins, 1947) and has also been recorded in man (Bethell, Gardiner and Mackinnon, 1939; Bethell, Blecha and Van Sant, 1943). However, in all these instances the anaemia was macrocytic in type, no doubt because the body gives priority to haemoglobin formation over other claims for amino-acids (Robscheit-Robbins, Madden, Rowe, Turner and Whipple, 1940; Whitby and Britton, 1950), so that a deficiency will be manifested in the cell stroma before haemoglobin formation is interfered with. Further, Robscheit-Robbins and Whipple (1949) have shown that casein (which was included not

only in the dried milk portion of all diets, but also as a direct supplement in half of them) is an excellent source of the amino-acids required for haemoglobin synthesis.

We may therefore exclude the possibility of protein deficiency and confine our attention to the haem moiety. From what has been said of the synthesis of this prosthetic group, possible causes of its failure are a deficiency of one of its precursors, namely protoporphyrin and iron, or of a catalyst such as copper or pyridoxin, or perhaps of hormones.

As has been indicated, the body is able to build up its own supply of protoporphyrin from comparatively simple molecules. It is difficult to conceive that a deficiency of simple metabolites such as acetic acid could develop, while, as has been pointed out above, any deficiency of the essential amino-acids concerned in porphyrin synthesis, such as glycine, would be likely to affect stroma formation before haemoglobin synthesis. Indeed, Hawkins, Sribhishaj, Robscheit-Robbins and Whipple (1931) have declared that there is no evidence that the pyrrhol nucleus is ever a limiting factor in the production of haemoglobin. On the other hand, Cartwright and Wintrobe (1948) have suggested that a deficiency of pyridoxin may lead to a failure in protoporphyrin synthesis, so that this possibility cannot be excluded.

Of the remaining causes of hypochromic microcytic anaemia, deficiencies of iron and copper are well established. Claims have also been made that deficiencies of certain other metals may sometimes be concerned, and hormonal disorders, though less well understood, should also be borne in mind; but no avitaminosis (other than pyridoxin deficiency) is known to cause this type of anaemia.

A deficiency of iron, however, is by far the most common of these causes, and its effects have been carefully studied both clinically and experimentally.

It is well recognised as a cause of 'nutritional anaemia' in young animals of many species, and the characters of the anaemia which it causes are similar to those observed in my experimental mice. For all of these reasons, then, I came to the conclusion that iron deficiency was the likeliest cause of the anaemia seen in Experiment 1; and that, while not excluding other possibilities from consideration, it would be prudent to begin by first examining the iron status of the experimental diets, and then investigating the possibility that the calcium carbonate supplement had interfered with the availability of iron.

2. EXPERIMENTAL PLANS

Spectrographic analysis* of the modified Sherman B diet used in Experiment 1 showed that it contained 35 parts of iron per million of fresh material. This rather low level in itself suggested that the relatively low haemoglobin values seen in the experimental animals on the basic diets might have been accountable to a simple insufficiency in iron intake. Accordingly, I decided to determine whether a supplement of iron would improve the haemoglobin concentration of the blood of breeding mice fed on the modified Sherman B diet.

Considering now the further degree of anaemia induced in Experiment 1 by the dietary supplements of calcium carbonate, some recorded evidence was found to suggest that dietary calcium hinders iron absorption or utilisation, but on the other hand many authors have found that it assists blood formation in iron deficiency (for a review of the literature, see p.220). In view of these conflicting reports, I decided also to determine whether a dietary supplement of iron would affect the anaemia associated with dietary supplements of calcium

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*Kindly performed by Dr. R. L. Mitchell, of the Macaulay Institute for Soil Research, Aberdeen.

carbonate in breeding mice. At the same time it would be possible to examine in more detail the pathology of the blood and the changes in the liver associated with these supplements, and also to learn whether the additional iron affected the reproductive performance of mated pairs.

Turning to the second angle of approach to the problem, it will be recalled that, in Experiment 1, the highest level of supplementary calcium carbonate had widened the Ca : P ratio of the diet from 0.7 : 1 to 2.3 : 1. While the first of these ratios is certainly narrow, the second might be considered rather wide - but not very greatly so, when it is remembered that the phosphorus content of the diet was as high as 0.48%. Nevertheless, this change from a narrow to a wide ratio is a considerable one, and it seemed worth while to ascertain whether it had exerted any influences on haemopoiesis or reproduction, either by inducing a deficiency of phosphate or by some other means. Reviewing the question of iron absorption, Cartwright (1947) has stated ".....all investigators are agreed that iron utilisation is affected by the Ca : P ratio in the diet but there is disagreement as to whether a high or a low ratio is more favorable." Accordingly, I decided also to determine experimentally whether restoration of the Ca : P ratio, by means of a phosphate supplement, would prevent the effects of supplementary calcium carbonate.

The effects of iron and phosphate supplements, in both the presence and the absence of each other, were therefore tested in the following experiment (Experiment 2), which also sought to confirm the findings of Experiment 1 in regard to the effects of a calcium carbonate supplement, and to provide further information on the pathology of the anaemia which it causes.

B. THE EFFECTS AND INTERACTIONS OF CALCIUM CARBONATE, IRON AND PHOSPHATE - EXPERIMENT 2

(Dr. Marion Richards collaborated with me in designing this experiment, and was responsible for keeping the breeding records and for recording the organ weights. She also supervised the feeding of the animals.

I was responsible for the remainder of the work described below, and, in particular, was entirely responsible for all haematological and pathological aspects of the studies.)

1. METHODS

(i) Construction of diets

The first question to settle was the choice of a basal diet. The basal diet used in Row 1 of Experiment 1 (i.e. the slightly modified Sherman B diet) at first seemed the obvious choice, because of its simplicity and because it had been the foundation diet from which the basal diets of the other rows had been evolved. However, although no statistically significant difference in any attribute had been detected between the means for the various rows, Dr. Richards believed that, so far as reproductive performance was concerned, the effects of the calcium carbonate supplement had been most marked in Row 4 (in which 5% of casein had been added to every 100 g. basal diet). The use of this diet as a basal diet might therefore offer some advantage in the reproductive trial by enhancing the differences between treatments. Since the blood examinations had indicated that the effects of a calcium carbonate supplement could be seen just as easily in Row 4 as in any other, there was - from the point of view of the blood studies to be made - no objection whatever to this course being followed.

Dr. Richards further believed that in Experiment 1 the reproductive performance of the mice on Col. 2 diets (with 0.5% of calcium carbonate) had been definitely superior to that of the animals on Col. 1 diets (with no

calcium carbonate) although again the differences between the columns had never quite reached statistically significant proportions. On the other hand, the haematological studies undertaken in Experiment 1 had suggested that Col. 1 might be the superior, but the evidence to support this view was rather meagre in view of the incompleteness of the data. Certainly there could be no doubt that, in all respects, the worst results had been given by the animals on Col. 4 diets. It was naturally desirable to create every opportunity for a significant result to be achieved in the proposed reproductive trial as well as in the haematological experiment; and on the basis of our experiences in Exp. 1, I considered that the haematological methods were sufficiently sensitive (provided a reasonable number of experimental animals were made available) to detect differences of the order of magnitude expected, and were certainly more sensitive than the criteria available for judging reproductive performance. On the whole, then, it seemed wise, in Exp. 2, to select the diet from Exp. 1, Col. 2, Row 4 (with 0.5% of calcium carbonate as the basal diet, and to use the corresponding diet in Col. 4 (with 2% of calcium carbonate)for comparison.

The diet selected as the basal diet (known as Diet 79) therefore had the following composition:

Wheat, whole ground	66 parts
Milk, full-cream, dried	33
Casein, unextracted	5
Sodium chloride	1
Calcium carbonate	0.5
	105.5 parts

Proximate analysis* showed that its composition was:

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* Kindly performed by Mr. J. Davidson of the Rowett Research Institute.

Moisture	14.0%
Crude protein	18.4
Crude fat	6.6
Nitrogen-free extract (by difference)	55.8
Crude fibre	1.2
Ash	4.0
	100.0%

The diet chosen for comparative purposes (known as Diet 81) had the same composition as Diet 79, except that the calcium carbonate content was increased (by the addition of a further 1.5 parts of the supplement to 105.5 parts of Diet 79) from 0.5 parts per 105.5 parts (or 0.47%) to 2 parts per 107 parts (or 1.87%). The difference in percentage calcium carbonate content between Diets 79 and 81 therefore amounted to 1.40%. As determined by chemical analysis*, the total calcium (Ca) content of Diet 79 was 0.515%, and the phosphorus (P) content 0.492%; the Ca : P ratio, therefore, was 1.05 : 1. The Ca content of Diet 81 was 1.069%, and the P content 0.485%, giving a Ca : P ratio of 2.20 : 1 (See Text Tables 18 and 19).

It was not altogether easy to decide how much additional iron should be fed, and in the end a rather arbitrary figure had to be selected. It was important that the amount be not too great, for, if it were to swamp the effects of the calcium carbonate, the point of the experiment would be lost. At the same time, if the anaemia were in fact due to iron deficiency then the amount of iron fed had to be sufficient to result in real improvement of the blood haemoglobin status. As the amount of iron which might be needed to overcome the effects of the supplement of calcium carbonate was of course quite

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* Kindly performed by Mr. J. Davidson, of the Rowett Research Institute.

TEXT TABLE 18.

Exp. 2. Plan of Diets

Supplement	Low $CaCO_3$	High $CaCO_3$
Nil	(Diet 79)	(Diet 81)
Fe		
P		
Fe + P		

Diet 79 contained 0.47% of $CaCO_3$.
Diet 81 contained 1.87% of $CaCO_3$.

Iron supplement (Fe): 10 p.p.m. Fe as ferric citrate.

Phosphate supplement (P): 0.57 g. P, as 2.87 g. sodium dihydrogen phosphate, per 105.5 g. of Diet 79 or 107 g. of Diet 81.

TEXT TABLE 19.

Exp. 2. Calcium and Phosphorus Contents of the Diets.

(For Diet Plan, see Text Table 18).

(a) Ca content (%)

0.515	1.069
0.515	1.069
0.502	1.041
0.502	1.041

(b) P content (%)

0.492	0.485
0.492	0.485
1.006	0.992
1.006	0.992

(c) Ca:P ratios (g.Ca/1g.P)

1.05	2.20
1.05	2.20
0.50	1.05
0.50	1.05

unpredictable, the problem resolved itself into finding the minimum amount of iron which could lead to an easily detectable increase in blood haemoglobin concentration.

It is difficult to lay down a precise figure for "normal requirements" of dietary iron, for any species, as so many factors are known to affect both the need for this element and its retention from the alimentary tract (see Cartwright, 1947). For the growing dog, Frost, Elvehjem and Hart (1940) believed that 10 mg. of soluble inorganic iron daily were somewhat in excess of the amount needed. In the human being, Davidson and Fullerton (1938) calculated that a total of about 725 mg. of iron are lost by a mother as a result of pregnancy and parturition; using this figure, Hynes (1948) has calculated the iron demands of a pregnant woman as averaging 3.8 mg. daily, whereas those of a normal man are given as 1.2 mg. Now, the basal diet fed to the mice would provide a total of fully 0.5 mg. of iron daily, on the basis of an average daily consumption of 15 g. of diet by a pregnant doe. Comparisons between species are always dangerous, more especially when there are great differences in their body weight and physiology; but it nevertheless seemed that even after making a conservative allowance for the heavy demands of multiple pregnancy, and also for much of the iron in the diet being unavailable, the basal modified Sherman B diet could not be deficient in iron to a gross extent. This view received indirect support from the originators of the diet: Sherman and Campbell (1924; 1929-30), who used their B diet to maintain rat colonies over several generations, found it eminently satisfactory for growth, reproduction, successful lactation, and longevity, and reported no untoward consequences. This being the case, I reasoned that a moderate level of additional iron should suffice to raise the blood haemoglobin to its physiological maximum.

Bearing this in mind, a considered guess was made, and it was decided that the level of supplementary iron (Fe) to be included in the diets should be 10 p.p.m. - equivalent to two-sevenths of the amount already in the basal ration. This was provided by adding 5.624 mg. of ferric citrate ($C_3H_4OH(COO)_3Fe.3H_2O$) to each 105.5 g. of Diet 79 and to each 107 g. of Diet 81 (see Text Table 18).

As regards the choice of a phosphate supplement, no objection could be seen to the use of one of the inorganic sodium phosphates. The sodium ion has no known toxic effect in the quantities it was proposed to employ, and, moreover, the phosphates of sodium are readily dissociable and contain a high proportion of phosphorus. Sodium di-hydrogen phosphate was the particular salt selected, first because it happened to be readily available, and second because, weight for weight, it not only contains less sodium but it provides a higher proportion of phosphate than the others, so that a smaller quantity of supplement would suffice.

The quantity of phosphate to be used was calculated as the amount which, when added to Diet 81, would just restore its Ca:P ratio to that of Diet 79 (see Text Tables 18 and 19). For every 107 g. of Diet 81, the required quantity of phosphorus was 0.57 g. This is contained in 2.87 g. of sodium di-hydrogen phosphate ($NaH_2PO_4.2H_2O$), which was therefore employed as the phosphate supplement per 107 g. of Diet 81. For control purposes, and also in order to examine the effect of a very narrow Ca : P ratio, a further diet was constructed in which the same quantity of phosphate was added to 105.5 g. of Diet 79.

By these means, it would now be possible to assess the effects of a phosphate supplement on diets which were probably only barely adequate, or perhaps mildly deficient, in iron. It was also of interest to know whether the effects of the supplement, if any, would be similar on diets more adequate in iron. Consequently, two further diets were constructed, in which Diets 79

and 81 were supplemented with both iron and phosphate in the same quantities as above (see Text Tables 18 and 19). In the event of both supplements proving to have effects of similar kind, these last two diets would also indicate whether the two supplements had any additive action. The Ca and P contents and the Ca : P ratios of all the diets are shown in Text Table 19.

(ii) Design (see also Technical Appendix, p.242)

On the advice of Mr. M. H. Quenouille, and in collaboration with Dr. Richards, I decided to employ eight replicates of a 2^3 factorial design. As described above, the dietary treatment variables were low or high levels of calcium carbonate ($CaCO_3$), and the presence or absence of supplementary iron (Fe) or phosphate (P), or both. From the plan of the diets in Text Table 18, it will be seen that the eight treatments could be considered not only individually but also as four horizontal pairs differing only in respect of the presence or absence of Fe and P, or as two vertical columns differing only in the level of calcium carbonate supplement. The treatments could also be grouped in other ways for comparison, e.g. the quartet containing added iron versus the quartet without added iron, or the quartet containing added phosphate versus the quartet without added phosphate; or, again, the pair containing iron and low calcium carbonate versus the pair containing iron and high calcium carbonate, and so forth. The design thus enabled the effect of treatments to be estimated either alone or in any combination, and also permitted the measurement of interactions.

The design called for sixty-four female mice, grouped in litters of four. These were distributed so that two litters were allotted to each block of eight dietary treatments, with the interactions $CaCO_3$ x Fe, $CaCO_3$ x P, Fe x P, and $CaCO_3$ x Fe x P partially confounded between litters. This arrangement of

TEXT TABLE 20.

Exp. 2. Arrangement of littermates.

(For Diet Plan, see Text Table 18)

BDEH LMQR	BCFG LNPS
ADFG KNQS	ACEH KMPR
ACFH KMPS	ADEG KNQR
BCEG LNPR	BDFH LMQS

Litters are designated A-S, omitting I, J and O.

littermates is illustrated in Text Table 20.

(iii) Procedure and technical methods

A general description and discussion of procedure and technical methods is given in the Technical Appendix, pp. 242 et seq. The procedure adopted in this experiment follows closely that general description, and only brief comments are necessary here.

From the stock mouse colony were bred sixty-four females, in groups of four littermates each, and sixty-four males. Each of the sixty-four female mice was introduced to its allotted experimental diet at 7 weeks of age, and one week later was mated with a male of similar age. The male was allowed to remain in the cage until the doe was pregnant for a second time. Full records of breeding performance were maintained by Dr. Richards. Three females which died within the first few days of the experiment were replaced, although in only one case was it possible to substitute a littermate.

For examination at the time of weaning (21 days of age), each litter was represented, where possible, by two males and two females; these were the two animals of each sex nearest to the median weight of the weanlings of that sex in the litter. The dams were examined on the day that their second litter weaned. Blood samples were taken under ether anaesthesia from the resected axillary vessels of each animal. Fresh blood was used for haemoglobin estimations (which were done by Haldane's method) and for blood films; for the other haematological procedures the blood was treated with oxalate anti-coagulant mixture.

Erythrocyte counts and packed cell volumes were estimated on the blood of all first litters and of the dams, and plain and supravitally stained blood films were made. Leucocyte counts were omitted. With second litters, only

haemoglobin concentration was determined, and then as much blood as possible was collected from every animal in one litter into a clean dry tube and allowed to clot. The serum was separated, transferred to fresh tubes fitted with rubber stoppers, and stored in a refrigerator for subsequent chemical analysis. Serum was also retained from the blood of the dams.

Heart weights were recorded from all animals, and thymus weights from second litter weanlings. The median lobes of the livers were stored in formol-saline for my subsequent histological examination.

2. RESULTS

(i) Haematological

The results, summarised for convenience, are presented in Text Tables 21, 23 and 24.

(a) ARRANGEMENT OF DATA

Beginning with first litters, the first step was to use the estimations of Hb, R.B.C. and P.C.V. to calculate, for each individual weanling, the values of M.C.H., M.C.H.C. and M.C.V. For the method of doing this, see the Technical Appendix, p. 257). Then, taking each of the six attributes in turn, its mean value for each of the 64 first litters was determined. There were eight missing first litters - seven where the litter had not survived, even in part, until weaning, and one where no litter had been born owing to an infertile mating; for these missing litters, estimates of the litter means were computed* by Mr. Quenouille. Making use of the arrangement of littermates within the design, Mr. Quenouille was also able to adjust any litter means which had been unduly influenced by littermate factors, but such adjustments as were necessary

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* By a similar method to that used in Exp. 1.

proved to be few and small. Finally, the litter means were used to find the mean value for each diet-group and its standard error. These diet-group means are the figures quoted in Text Table 21. Owing to the fact that one or more estimated observations on missing litters were included in some of these means, their standard errors were not all identical; mean standard errors were therefore calculated for each attribute, and these are also shown in Text Table 21.

In exactly the same way, diet-group means and mean standard errors were calculated for the Hb of second litters (seven of which were missing and had to be estimated), and for all the haematological attributes of the dams. These values are listed in Text Tables 23 and 24.

Effects

The difference between any pair of diet-group means constitutes the effect on the attribute in question of the variation in dietary treatment. It must be remembered that strictly speaking these differences were not the true effects, but merely experimental estimates of the true effects, and if they are to have real meaning they must be accompanied by some indication of how accurate the estimate is likely to have been. This is accomplished by means of the appropriate standard error, which is used to calculate the significance of an effect.

The simple effects of the various treatments are obvious from inspection of the data, e.g. the addition of the higher level of calcium carbonate to the otherwise unsupplemented diet reduced the Hb of first litters from 5.00 to 2.66 g./100 ml., reduced the R.B.C. of first litters from 5.4 to 3.4 millions/cu.mm., and so forth (Text Table 21). The significance of such effects (in the above examples, 2.34 and 2.0 respectively) can be determined in each case by using the mean standard error applicable to comparisons between two

diet-group means, i.e. the means of two sets of eight litters each. For first litters' Hb and R.B.C. these are ± 0.63 and ± 0.50; both differences are three or four times as large as their respective standard errors, and, when examined by the t test, are seen to be highly significant. The significance of differences between any two treatments can be determined in this way.

As stated earlier, the factorial design of the experiment permitted comparisons to be drawn not only between individual diet-groups but also between various combinations of diet-groups. This amounts to estimating the response to a particular dietary treatment by averaging its effects over other treatments. Such an estimate of effect is more accurate because it is based on a larger number of observations, is more sensitive because the standard error is smaller, and is valid provided that there are no important interactions between treatments - that is to say, that the effect is the same whether other dietary supplements are present or not (see later). For instance, the overall average effect of iron on first litters' Hb can be obtained by subtracting the mean of all diet-groups not containing iron from the mean of all those that do contain it. When this is done we get

$$\tfrac{1}{4}(5.93 + 4.45 + 6.87 + 4.00) - \tfrac{1}{4}(5.00 + 2.66 + 4.71 + 3.39) = 1.37.$$

This difference is more than four times its standard error (i.e. the standard error of differences between means of sets of thirty-two litters, namely ± 0.31) and the difference is therefore very highly significant ($P < 0.001$). When the overall effect of the higher level of calcium carbonate on first litters' Hb is determined in this way, it is seen to be more than six times its standard error ($2.00 > 6 \times 0.31$).

Comparisons between means of sixteen litters can be made on the same principle. For instance, the effect on first litters' Hb of iron in the

absence of phosphate is

$$\tfrac{1}{2}(5.93 + 4.45) - \tfrac{1}{2}(5.00 + 2.66) = 1.36.$$

The standard error applicable to this comparison (a comparison between the means of sets of sixteen litters) is ± 0.44. Here again the effect is highly significant ($P < 0.01$) as it is about three times as great as its standard error.

Interactions

The design of the experiment also permitted the measurement of interactions between dietary treatments. These interactions were not only interesting in themselves, but also had to be known in order to validate the method, described above, of averaging the effects of one dietary treatment over other treatments.

By interaction is meant a change in the magnitude or direction of the effect of a treatment, as a result of the presence of some other treatment or treatments. When two treatments interact, the extent of the interaction represents the deviation, one way or the other, from the additivity which would otherwise have been expected. Three two-factor interactions ($CaCO_3$ x Fe, $CaCO_3$ x P, and Fe x P) could be measured, and also one three-factor interaction ($CaCO_3$ x Fe x P). For instance, the interaction $CaCO_3$ x Fe describes the change in the effect exerted by the higher level of calcium carbonate as a result of the additional presence of iron; or, equally, the change in the effect of iron as a result of the additional presence of the higher level of calcium carbonate. The presence or absence of the third variable - in this example, phosphate - is not considered in two-factor interactions, the effects of the other two being averaged over it.

The interaction $CaCO_3$ x Fe is computed by first finding the mean effect of high $CaCO_3$ (i.e. the difference between the values given by high $CaCO_3$

and low $CaCO_3$) in the two pairs of diet-groups containing Fe. The same thing is then done with the two pairs of diet-groups not containing Fe. We now have two mean effects - one of high $CaCO_3$ in the presence of Fe, the other of high $CaCO_3$ in the absence of Fe. By subtracting the latter from the former, and halving the difference in order to restore it to unit level, we get a measure of the effect of the presence of Fe on the effect of high $CaCO_3$, i.e. The interaction $CaCO_3$ x Fe.

In the case of first litters' Hb, therefore, the interaction $CaCO_3$ x Fe was

$$\frac{1}{2}\left\{ \frac{(4.45 - 5.93) + (4.00 - 6.87)}{2} - \frac{(2.66 - 5.00) + (3.39 - 4.71)}{2} \right\}$$

$$= \frac{1}{2}\left\{ (-2.17) - (-1.83) \right\} = -0.17$$

The meaning of this result is that in diet-groups containing both high $CaCO_3$ and Fe the deviation from the expected additivity of the effects of these supplements proved to be -0.17; so that, in this experiment, the effect of high $CaCO_3$ was changed in the presence of Fe by -0.17, i.e. it became more negative.

An interaction Fe x $CaCO_3$ would describe the effect of high $CaCO_3$ on the effect of Fe. Since the deviation from the expected additivity is the same with this interaction as with $CaCO_3$ x Fe, it must have the same magnitude and need not therefore be considered separately. It would be given by

$$\frac{1}{2}\left\{ \frac{(4.45 - 2.66) + (4.00 - 3.39)}{2} - \frac{(5.93 - 5.00) + (6.87 - 4.71)}{2} \right\}$$

and this, clearly, must also be - 0.17.

The significance of the interaction must now be considered. The interaction is based on 64 litter-means, so that its standard error is the same as the standard error applicable to the difference between two means each

TEXT TABLE 21.

Exp. 2. 1st litters. Diet-group means, standard errors, effects and interactions for haematological attributes

Dietary Supplement	Hb (g./100 ml.)		R.B.C. (10^6/cu.mm.)		P.C.V. (%)		M.C.H. (μμg.)		M.C.H.C. (%)		M.C.V. (cu.μ)	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	5.00	2.66	5.4	3.4	21.5	13.7	9.4	7.6	23.0	18.2	41.2	41.4
Fe	5.93	4.45	5.9	5.2	24.5	20.4	10.0	8.5	24.0	21.5	41.6	39.9
P	4.71	3.39	5.5	4.3	20.8	16.4	8.6	7.7	22.5	19.9	38.2	38.6
Fe + P	6.87	4.00	6.5	4.3	27.4	18.6	10.6	9.1	25.1	20.8	42.6	43.8
Standard error of the differences between means of:												
32 litters	± 0.31		± 0.25		± 1.05		± 0.40		± 0.79		± 1.13	
16 litters	± 0.44		± 0.36		± 1.48		± 0.57		± 1.11		± 1.60	
8 litters	± 0.63		± 0.50		± 2.10		± 0.80		± 1.57		± 2.27	
Overall effect of:												
High $CaCO_3$	- 2.00^{xxx}		- 1.53^{xxx}		- 6.26^{xxx}		- 1.44^{xxx}		- 3.57^{xxx}		- 0.03	
Fe	+ 1.37^{xxx}		+ 0.83^{xx}		+ 4.63^{xxx}		+ 1.24^{xx}		+ 1.95^{x}		+ 2.09	
P	+ 0.22		+ 0.18		+ 0.78		+ 0.07		+ 0.40		- 0.22	
Interactions:												
$CaCO_3$ x Fe	- 0.17		+ 0.1		- 0.2		- 0.1		+ 0.2		- 0.3	
$CaCO_3$ x P	- 0.07		- 0.2		- 0.3		+ 0.2		+ 0.2		+ 0.1	
Fe x P	+ 0.01		- 0.3		- 0.2		+ 0.5		- 0.2		+ 2.67^{x}	
$CaCO_3$ x Fe x P	- 0.60		- 0.56^{x}		- 2.0		- 0.2		- 1.0		+ 0.7	

Each diet-group comprised eight litters.

For all comparisons, t is 2.02 when P is 0.05.

^{x}Significant ($P < 0.05$)

^{xx}Significant ($P < 0.01$)

^{xxx}Significant ($P < 0.001$)

based on 32 litter-means, i.e. ± 0.31. The magnitude of the interaction is thus only about one-half of its standard error, and so it could easily have arisen by chance and is therefore not significant. (To reach significance at the 5% level the interaction would have had to be about 2.1 times as large as its standard error). In this experiment, then, there was no significant interaction $CaCO_3$ x Fe on Hb.

In a similar manner, the interaction $CaCO_3$ x Fe can be measured on each of the remaining five haematological attributes examined. This was done, and then the other two-factor interactions were each, in turn, determined on all six attributes. The results of these procedures are presented in Text Table 21. The dams' data were treated in exactly the same way; the results are shown in Text Table 24.

The remaining interaction calling for consideration is the three-factor interaction $CaCO_3$ x Fe x P. This interaction describes a difference between the mean effect of one of the treatments in the presence and in the absence of both the other two, as compared with its mean effect in the presence of these two treatments separately. This interaction, too, was determined on all six haematological attributes for both first litters and dams (Text Tables 21 and 24). As an example of the three-factor interaction, that for the R.B.C. of first litters (Text Table 21) is given by

$$\tfrac{1}{2}\left\{\frac{(4.28 - 6.51) + (3.40 - 5.36)}{2} - \frac{(5.15 - 5.92) + (4.31 - 5.48)}{2}\right\}$$

$$= \tfrac{1}{2}\left\{(-2.10) - (-0.97)\right\} = 0.56, \pm 0.25$$

The interpretation of this interaction will be discussed at a later stage.

(b) FIRST LITTERS

As has been explained above, the most sensitive effects are obtained by averaging them over all other treatments, but before this procedure can be

valid it must be determined that there are no important interactions. Two interactions reached the 5% level of significance with first litters' data (namely, Fe x P on M.C.V. = 2.67 ± 1.13, and $CaCO_3$ x Fe x P on R.B.C. = -0.56 ± 0.25), and will be discussed below in some detail.

The interaction Fe x P on M.C.V. is

$$\frac{1}{2}\left\{\frac{(42.6-38.2)+(43.8-38.6)}{2} - \frac{(41.6-41.2)+(39.9-41.4)}{2}\right\}$$

$$= \frac{1}{2}\left\{(+4.8)-(-0.55)\right\} = +2.67, \quad \pm 1.13$$

and as it is more than 2.1 times its standard error, it indicates that in this experiment the presence of P significantly changed the effect of Fe on M.C.V. ($P < 0.05$). Since its sign is positive, the interaction shows that in the presence of P the effect of Fe was significantly greater than would have been expected; or, equally, that in the presence of Fe the effect of P was significantly greater than would have been expected.

The effect of Fe on M.C.V. has thus been shown to be not independent of the presence of another treatment. If this finding can be accepted (see later), it would mean that to judge the effect of Fe by averaging its effects over all other treatments would not be a valid procedure, and would tell only part of the truth. In order to obtain more accurate and complete information on its action, we have to consider separately the effects of Fe in the presence and in the absence of the interacting factor. On doing this, we find that the effect of Fe in the presence of P was itself significantly positive; as we have seen above it was +4.8 ± 1.60 (the standard error, of course, being that applicable to differences between means of 16 litters). In the absence of P the effect of Fe was -0.55 ± 1.60, but this effect is not significantly different from zero. Thus, we could say that Fe in the presence of P significantly increased the M.C.V., but in the absence of P it had no significant effect.

Similarly, the overall average effect of P would not truly reflect its

TEXT TABLE 22.

Exp. 2. First litters. Effects contributing to the interaction $CaCO_3$ x Fe x P on R.B.C.

In the presence of	Standard error ±	Effect of:		
		$CaCO_3$	Fe	P
None	0.50	-1.96	+0.5	+0.1
High $CaCO_3$	0.50	...	+1.8^x	+0.9
Fe	0.50	-0.77	...	+0.6
P	0.50	-1.17^x	+1.0	...
High $CaCO_3$ + Fe	0.50	...	...	-0.9
High $CaCO_3$ + P	0.50	...	0.0	...
Fe + P	0.50	-2.23^x	...	...
Mean: presence & absence of both others	0.36	-2.10^x	+0.25	-0.40
Mean: both others separately	0.36	-0.97^x	+1.40^x	+0.75^x
INTERACTION (= half the difference of the preceding two means)	0.25	-0.56^x	-0.56^x	-0.56^x

^{x} Significant ($P < 0.05$) When P is 0.05, t is 2.02.

action. Considering its mean effects in the presence and absence of the interacting treatment Fe, we find that they were, respectively, + 2.45 ± 1.60 and -2.90 ± 1.60. Neither of these is itself significantly different from zero, although, of course, the magnitude of the interaction has shown that they are significantly different from each other. All we can conclude here is that the effect of P was more positive in the presence than in the absence of Fe.

The other significant interaction, $CaCO_3$ x Fe x P on R.B.C., was calculated on p. 57 and shown to be $\frac{1}{2}\{(-2.10) - (-0.97)\}$, that is -0.56. Since the magnitude of the interaction just exceeds 2.1 times its standard error (± 0.25) the interaction is significant ($P < 0.05$); that is to say, the effect of high $CaCO_3$ in the presence or absence of both Fe and P (-2.10 ± 0.36) was significantly more negative than was its mean effect in the presence of Fe and P separately (-0.97 ± 0.36). Both these effects, however, are themselves significantly different from zero ($P \leq 0.001$, < 0.05), so that it is still true to say that the effect of high $CaCO_3$ was negative, no matter in what combination of treatments it was fed: but it was significantly stronger in some combinations than in others.

In the same way, the interaction could be interpreted to mean that the effects of Fe were more negative in the presence and absence of both the other two treatments than in the presence of these treatments separately; and the same could also be said of the effects of P. Effects were therefore calculated for each treatment in the presence of either, neither, and both of the others, and are listed in Text Table 22. From this Table it can be seen that the mean effect of Fe in the presence and absence of both other treatment variables was not significantly different from zero; the same was also true in the case of P. But, again in both cases, the mean effect of the

supplement reached significance when it was fed in the presence of the other variables separately, and these significant effects were both positive.

The two interactions which reached the 5% level of significance have been discussed above in some detail. This has been done mainly to provide examples of significant two-factor and three-factor interactions, and - so that further detailed explanation will not be necessary when interactions are again encountered - to demonstrate the method used in calculating them and the ways in which their meaning can be interpreted. However, before an interaction is accepted, it is as well to consider its importance against the background of the experiment as a whole. Significance at the 5% level provides that an interaction of such magnitude would not be attained, on average of a big number, more often than once in twenty times as the result of chance alone. Now, out of the twenty-four interactions tested (four on each of the six attributes) only two reached significance at the 5% level. This is, then, a proportion that need occasion no surprise, particularly since, although these twenty-four were not entirely independent of one another, the two that reached the 5% level of significance had little apparent relation to each other. Furthermore, in marked contrast to the situation with the overall effects, which were in the main remarkably consistent, with the interactions there was little evidence of consistency in direction and magnitude over the various attributes. (A possible exception is the three-factor interaction, which in five of the six cases was negative and moderately large.) The only conclusion that can be reached is that these interactions, if in fact they existed at all, were small; and, in comparison with the magnitude of the main overall effects, unimportant. It is true that they should be, and will be, borne in mind when finally interpreting the results of this experiment, but they cannot constitute a real objection to the principle of averaging effects over all other treatments.

FIGURE 21

Exp. 2. Litters. Blood Smear

High calcium carbonate diet-group. Showing normoblasts and reticulocytes. (Brilliant cresyl blue supravitally and Leishman; x 1500)

The overall effects of the supplements may therefore be considered valid. The magnitude, direction and significance of the effects are set out below each attribute in Text Table 21, and may be interpreted quite simply. The higher level of calcium carbonate reduced the values of Hb, R.B.C., P.C.V., M.C.H. and M.C.H.C., the effects being highly significant ($P < 0.001$) in every instance. Supplementation of the diet with iron also produced significant effects on all these attributes, particularly Hb and P.C.V. ($P < 0.001$), but in the opposite direction. In striking contrast were two findings: first, that none of the dietary treatments could be shown to produce a significant effect on M.C.V.; and second, that the effects of the phosphate supplement never approached significance.

On the evidence of the overall effects, then, the higher level of calcium carbonate in the mother's diet induced a hypochromic, normocytic anaemia in weanlings of the first litters; and weanlings from the diet-groups without added iron also exhibited hypochromic anaemia when compared with those from the diet-groups that did contain it.

The appearance of the red blood cells in films stained by Leishman's method confirmed these findings. In anaemic animals the mature erythrocytes were hypochromic, and anisocytosis and poikilocytosis were common. Examination of the supravitally stained smears (Fig. 21) showed that reticulocytes and normoblasts were numerous in all specimens. Although systematic differential red cell counts were not made on all smears, immature cells appeared to be rather more numerous in the blood of weanlings from the high calcium carbonate groups. In these animals intermediate and early normoblasts were encountered, sometimes in considerable numbers.

(c) SECOND LITTERS

The summarised results of Hb estimations on the blood of second litters

TEXT TABLE 23.

Exp. 2. First and second litters. Diet-group means, standard errors, and effects on Hb (g./100 ml.).

Dietary Supplement:	First litters		Second litters	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	5.00	2.66	5.77	3.12
Fe	5.93	4.45	7.76	4.53
P	4.71	3.39	6.57	3.70
Fe + P	6.87	4.00	8.52	5.98
Standard error of the differences between means of:				
32 litters	± 0.31		± 0.40	
16 litters	± 0.44		± 0.56	
8 litters	± 0.63		± 0.80	
Overall effect of:				
High $CaCO_3$	- 2.00^{xxx}		- 2.83^{xxx}	
Fe	+ 1.37^{xxx}		+ 1.91^{xxx}	
P	+ 0.22		+ 0.90^{x}	

Each diet-group comprised eight litters.

For all comparisons, t is 2.02 when P is 0.05.

^{x} Significant effect ($P < 0.05$).

^{xxx} Significant effect ($P < 0.001$).

For comparisons within first litters, t = [illegible]

For comparisons within second litters, t = [illegible]

are shown in Text Table 23, which includes for comparison the corresponding figures for first litters. The data were treated in the same manner as were those of first litters; there were seven missing observations which had to be estimated.

The interactions all proved to be not significantly different from zero, so that effects could be averaged over other treatments. As can be seen from the Table, the negative overall effect of the higher level of calcium carbonate was again very pronounced ($P < 0.001$), as was the positive effect of iron ($P < 0.001$). This time the effect of phosphate was also significantly positive ($P < 0.05$), although it was small compared with those of calcium carbonate and iron. It can therefore be concluded that the higher level of calcium carbonate in the dams' diet induced anaemia in their second litters at weaning, while iron and phosphate supplements had the reverse effect.

It can also be seen from Text Table 23 that, compared with that of first litters, second litters' Hb was improved in every diet-group. This improvement is highly significant ($P < 0.01$).

(d) DAMS

The summarised results of the dams' haematological measurements are presented in Text Table 24. The data were treated in the same manner as were those of first litters, and effects and interactions were also studied similarly. Three out of the twenty-four interactions were significant at the 5% level - one of these being significant also at the 1% level - and a fourth was on the borderline of significance (Text Table 24). Although one or two might have been the result of chance, it is most unlikely that all were. In considering the results, then, it will be necessary to take these interactions into account.

The differences were not always so large as those seen with the litters,

TEXT TABLE 24.

Exp. 2. Dams. Diet-group means, standard errors, effects and interactions for haematological attributes.

Dietary Supplement	Hb (g./100 ml.)		R.B.C. (10^6/cu.mm.)		P.C.V. (%)		M.C.H. (μμg.)		M.C.H.C. (%)		M.C.V.(cu.μ)	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	11.93	9.83	9.7	8.5	42.6	37.5	12.4	11.4	28.0	25.7	44.2	44.4
Fe	14.12	12.70	10.6	10.0	51.6	43.6	13.5	12.7	27.6	28.8	48.9	43.5
P	14.83	10.74	10.5	9.9	51.1	40.2	14.4	10.8	29.2	26.7	49.0	40.4
Fe + P	14.43	12.79	10.2	9.4	47.9	44.9	14.1	13.8	30.2	28.6	46.9	48.1
Standard error of the differences between means of:												
32 animals	± 0.52		± 0.39		± 1.7		± 0.44		± 0.55		± 1.1	
16 animals	± 0.74		± 0.55		± 2.4		± 0.62		± 0.78		± 1.6	
8 animals	± 1.04		± 0.78		± 3.4		± 0.88		± 1.10		± 2.2	
Overall effect of:												
High $CaCO_3$	- 2.32^{xxx}		- 0.82^{x}		- 6.8^{xxx}		- 1.45^{xxx}		- 1.28^{x}		- 3.2^{xx}	
Fe	+ 1.67^{xx}		+ 0.42		+ 4.1^{x}		+ 1.28^{xx}		+ 1.39^{x}		+ 2.3^{x}	
P	+ 1.07^{x}		+ 0.32		+ 2.2		+ 0.77		+ 1.09		+ 0.8	
Interactions:												
$CaCO_3$ x Fe	+ 0.78		+ 0.1		+ 1.2		+ 0.8		+ 1.13^{x}		+ 1.0	
$CaCO_3$ x P	- 0.55		+ 0.1		- 0.2		- 0.5		- 0.8		- 0.5	
Fe x P	- 0.85		- 0.80^{x}		- 3.4^{x}		+ 0.1		+ 0.1		+ 0.5	
$CaCO_3$ x Fe x P	+ 0.44		- 0.2		+ 2.6		+ 0.8		- 0.6		+ 3.8^{xx}	

Each diet-group comprised eight animals.
For all comparisons, t is 2.01 when P is 0.05.

^{x}Significant effect ($P < 0.05$)
^{xx}Significant effect ($P < 0.01$)
^{xxx}Significant effect ($P < 0.001$)

but the directions of the overall effects were essentially similar. In the case of Hb, the negative effect of the higher level of calcium carbonate was very strong, being more than four times its standard error ($P \ll 0.001$). The iron effect was also marked ($P < 0.01$) and, as with the litters, positive in direction. A positive phosphate effect also appeared and just reached significance ($P < 0.05$). (Since with the dams there were fewer missing observations and therefore more degrees of freedom, differences attained significance at the 5% level when only 2.01 times as large as their standard error.) There were no significant interactions on Hb.

The overall calcium carbonate effect on R.B.C. was significant ($P < 0.05$), and could be considered valid because all the interactions involving $CaCO_3$ were insignificant. While neither the iron nor the phosphate overall effects were significant, they can be discounted by the significantly negative ($P < 0.05$) Fe x P interaction, which means that the value of R.B.C. was less in those diet-groups where Fe and P were either both absent or both present than in those which contained one or the other. Indeed, the effect of Fe in the absence of P proved to be significantly positive, as was that of P in the absence of Fe (Text Table 25 (a)).

A very strong and significant ($P \ll 0.001$) negative overall effect with calcium carbonate was apparent in the P.C.V. The iron and phosphate effects, however, must be considered in relation to the Fe x P interaction, which proved to be negative and on the borderline of significance ($P \doteq 0.05$). From Text Table 25(b) we see that the effect of Fe in the absence of P, and that of P in the absence of Fe, were both significantly positive. As with the R.B.C., iron and phosphate separately both had positive effects which were countered, in each case, by the presence of the other.

With M.C.H., strong negative calcium carbonate and positive iron effects were again apparent; the effect of phosphate was not significant, nor were

TEXT TABLE 25.

Exp. 2. Dams. Effects contributing to significant interactions.

(a) Fe x P on R.B.C.

In the presence of:		Standard error ±	Effect of: Fe		Effect of: P	
Fe	High $CaCO_3$	0.78	...		-0.6	
	Low $CaCO_3$	0.78	...		-0.4	
	Mean	0.55		...		-0.5
P	High $CaCO_3$	0.78	-0.5		...	
	Low $CaCO_3$	0.78	-0.3		...	
	Mean	0.55		-0.4		...
Neither	High $CaCO_3$	0.78	+1.5		+1.4	
	Low $CaCO_3$	0.78	+0.9		+0.8	
	Mean	0.55		$+1.2^{x}$		+1.1
INTERACTION (= half difference of means)		0.39		-0.8^{x}		-0.8^{x}

(b) Fe x P on P.C.V.

In the presence of:		Standard error ±	Effect of: Fe		Effect of: P	
Fe	High $CaCO_3$	3.4	...		+1.3	
	Low $CaCO_3$	3.4	...		-3.7	
	Mean	2.4		...		-1.2
P	High $CaCO_3$	3.4	+4.7		...	
	Low $CaCO_3$	3.4	-3.2		...	
	Mean	2.4		+0.7		...
Neither	High $CaCO_3$	3.4	+6.1		+2.7	
	Low $CaCO_3$	3.4	$+9.0^{x}$		$+8.5^{x}$	
	Mean	2.4		$+7.5^{xx}$		$+5.6^{x}$
INTERACTION (= half difference of means)		1.7		-3.4		-3.4

(continued)

(continued)

TEXT TABLE 25.

Exp. 2. Dams. Effects contributing to significant interactions.

(c) $CaCO_3$ x Fe on M.C.H.C.

In the presence of:		Standard error ±	Effect of: High $CaCO_3$	Effect of: Fe
High $CaCO_3$	P present	1.10	...	+1.9
	P absent	1.10	...	$+3.1^{x}$
	Mean	0.78	...	^{xx} +2.5
Fe	P present	1.10	-1.6	...
	P absent	1.10	+1.2	...
	Mean	0.78	-0.2	...
Neither	P present	1.10	-2.5^{x}	+1.0
	P absent	1.10	-2.3^{x}	-0.4
	Mean	0.78	^{xx} -2.4	+0.3

(d) $CaCO_3$ x Fe X P on M.C.V.

In the presence of:	Standard error ±	Effect of: High $CaCO_3$	Effect of: Fe	Effect of: P
None	2.2	+0.2	$+4.7^{x}$	$+4.8^{x}$
High $CaCO_3$	2.2	...	-0.9	-4.0
Fe	2.2	-5.4^{x}	...	-2.0
P	2.2	-8.6^{xx}	-2.1	...
High $CaCO_3$ + Fe	2.2	...	...	$+4.6^{x}$
High $CaCO_3$ + P	2.2	...	$+7.7^{xx}$	...
Fe + P	2.2	+1.2	...	...
Mean: presence and absence of both others	1.6	+0.7	$+6.2^{xx}$	$+4.7^{x}$
Mean: both others separately	1.6	-7.0^{xxx}	-1.5	-3.0
INTERACTION (= half the difference of the preceding two means)	1.1	$+3.8^{xx}$	$+3.8^{xx}$	$+3.8^{xx}$

When P is 0.05, t is 2.01.

^{x}Significant ($P < 0.05$)
^{xx}Significant ($P < 0.01$)
^{xxx}Significant ($P < 0.001$)

there any significant interactions.

On M.C.H.C., the overall effects of calcium carbonate and iron were both significant, but there was also an interaction, which was just significant, between these two treatments. This showed that the effects of each were more positive in the presence than in the absence of the other; these effects are shown in Text Table 25(c). The overall effect of phosphate on M.C.H.C. was positive, and not affected by interactions; it was on the borderline of significance ($\underline{P} \doteq 0.05$).

The pattern of the overall effects was similar with the M.C.V., high calcium carbonate having, apparently, a strongly significant overall lowering effect ($\underline{P} < 0.01$), and iron having a significant overall augmenting effect ($\underline{P} < 0.05$). The overall effect of phosphate was not significant, nor were any of the two-factor interactions. However, the three-factor interaction proved to be positive and strongly significant ($\underline{P} \ll 0.01$) with this attribute, so that the mean effect of any one of the three treatments was more positive when the other two were both present or both absent than when they were present separately (Text Table 25(d)). Indeed, the effect of high $CaCO_3$ was negative only in the separate presence of Fe or P, and the effects of Fe and of P were positive only in the presence or in the absence of both the other two interacting treatments; but in every case these effects were strong - so strong, in fact, that they themselves reached a level significantly different from zero despite the fact that the comparisons involved only eight animals on each side.

With the dams, then, the general picture of the effects of the supplements on haematological attributes was the same as that seen in the litters, although it was sometimes complicated by interactions between treatments. The higher level of calcium carbonate induced an anaemia, while iron and phosphate both had the reverse effect.

The stained films of dams' blood presented the same features as those of

weanlings' blood, although the abnormalities were on the whole less pronounced. Supravitally stained blood films prepared from dams in all diet-groups showed the presence of many immature forms of red cells, but they differed from those of the litters in that very young normoblasts were seldom encountered.

(e) CONCLUSIONS

The higher level of calcium carbonate in the dams' diet induced anaemia in the litters as well as in the dams themselves. In both classes of animal, but especially in the litters, the anaemia was hypochromic, while with the dams it was also microcytic. The supplement of iron had a positive effect on the values of all the litters' attributes except M.C.V.; the phosphate supplement, however, in general had no apparent effect on litter attributes, although with second litters it increased the Hb values. The interactions Fe x P on M.C.V. and $CaCO_3$ x Fe x P on R.B.C. were respectively positive and negative with first litters, but their significance is doubtful. Iron also acted in the same way with the attributes of the dams' blood, and, in the case of Hb, phosphate also had a positive effect; but these general statements must be qualified by four interactions which reached a significant level, namely, Fe x P on R.B.C. and P.C.V. (both negative), $CaCO_3$ x Fe on M.C.H.C. (positive) and $CaCO_3$ x Fe x P on M.C.V. (strongly positive). In both litters and dams the main findings were confirmed in blood films by the morphological characteristics of the cells of the erythroid series.

(ii) Histological

Frozen sections of the median lobe of the liver of each mother and of a representative of each first litter were stained for fat and examined. (Where all animals in the first litter had succumbed before weaning, the livers of second litters were used instead.) The procedure described under Exp. 1 was adopted

TEXT TABLE 26.

Exp. 2. First litters and dams. Diet-group means and standard errors for quantity of visible fat (assessed 0-4 histologically), and the separate effects of the dietary supplements.

Dietary supplement	First litters		Dams	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	1.6	2.5	2.0	1.2
Fe	0.9	1.9	2.0	2.1
P	1.6	1.6	1.6	2.1
Fe + P	0.8	2.1	1.0	1.9
Standard error of the differences between means of:				
32 animals	± 0.26		± 0.24	
16 animals	± 0.37		± 0.35	
8 animals	± 0.53		± 0.49	
Overall effect of:				
High $CaCO_3$	+ 0.81^{xx}		+ 0.18	
Fe	- 0.44		+ 0.08	
P	- 0.19		- 0.15	

Each diet-group comprised eight animals.

For all comparisons, t is 2.0 when P is 0.05.

^{xx} Significant effect ($P < 0.01$).

to assess the amount of visible fat in each of the liver sections. Objectivity in assessment was achieved by labelling each slide with a code number which gave the observer no clue to the identity of the specimen, and, in the case of the dams' livers, by recording assessments by two independent observers.*

The results are presented in Text Table 26. The effect of the higher level of calcium carbonate in the dams' diet was to increase the amount of visible fat in the livers of weanlings ($P < 0.01$), and there was a suggestion that the iron supplement may have had the opposite effect ($0.1 > P > 0.05$). The effect of the phosphate supplement was not significant, nor were there any dietary effects on the visible fat in the livers of the dams. Interactions were in no instance significant.

With the litters, the essential lesion in all the affected livers was fatty degeneration, similar to that seen in Exp. 1. Photomicrographs of livers from several diet-groups are reproduced in Figs. 13-16 and 22-25, which show also the centrilobular distribution of the fatty changes. In the dams' livers the changes were often more diffuse, and in many cases the hepatic cells did not exhibit a comparable degree of damage. Quite evidently the presence of large amounts of fat in the mothers' livers was due, mainly at least, to some factor other than the dietary supplements.

(iii) Reproduction data

One of the does on diet 81 (i.e. high $CaCO_3$ only) failed to produce a litter. When the experiment was otherwise complete, she was re-mated with a male which was known to be fertile, and immediately conceived. In view of her age at that time, however, data relative to this animal have not been included

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* Mr. E. A. S. Rattray, of the Pathology Department, Rowett Research Institute, kindly made the second observations.

FIGURES 22-23

Exp. 2. Livers of weanling mice

Stained for fat (shows dark) by Scharlach R and haemalum

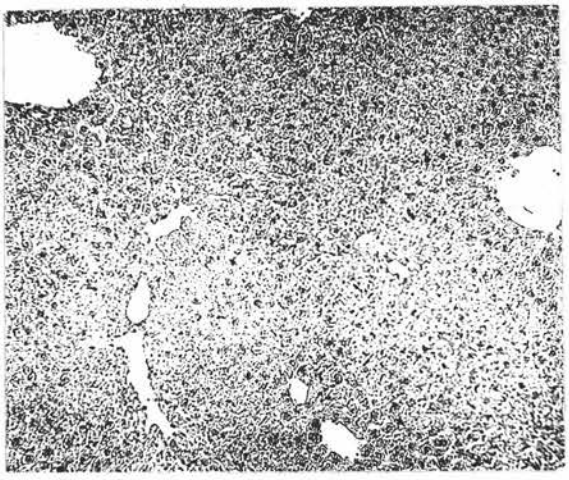

Fig. 22. Low calcium carbonate plus iron. (x 80)

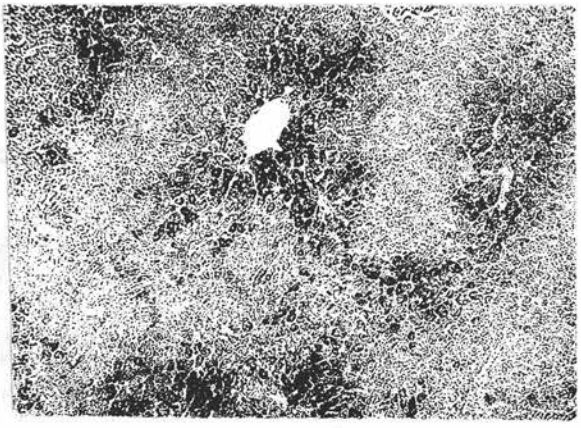

Fig. 23. Low calcium carbonate plus phosphate (x 80)

FIGURES 24-25

Exp. 2. Livers of Weanling Mice

Stained for fat (shows dark) by Scharlach R and haemalum

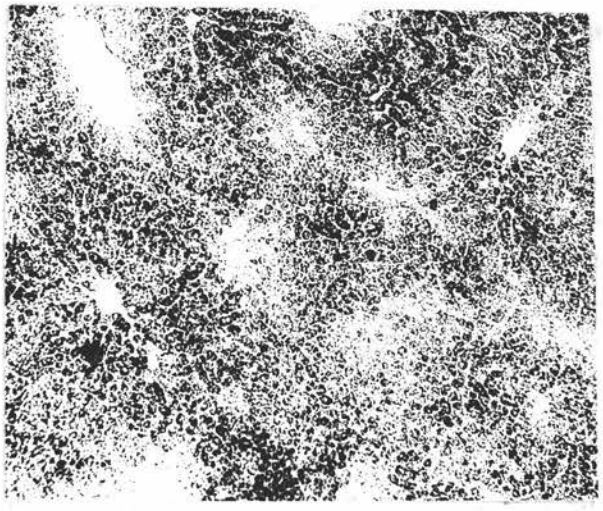

Fig. 24. High calcium carbonate plus iron plus phosphate (x 80)

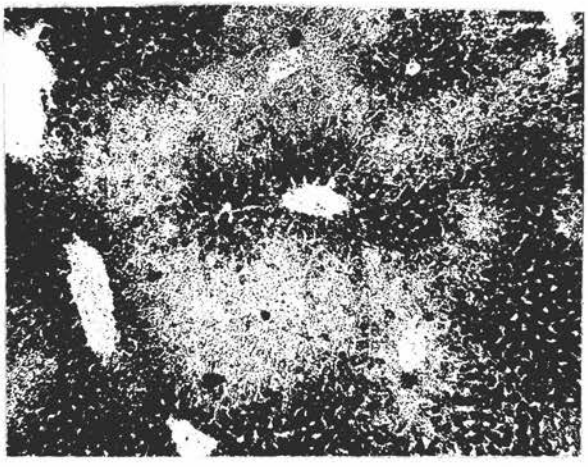

Fig. 25. High calcium carbonate plus phosphate. (x 80)

in the experimental records. All other does bore two litters.

Of the remaining 31 does in the high $CaCO_3$ diet-groups, six failed to rear any of their first litter young to weaning age; while, of the 32 does in the low $CaCO_3$ diet-groups, all but one weaned at least some of their first litter offspring. These seven animals, however, all succeeded in rearing some members of their second litters, but the entire second litters of two other dams in the high $CaCO_3$ diet-groups, and of four others in the low $CaCO_3$ diet-groups, all succumbed before weaning.

The data on reproduction are presented in Text Tables 27-29, which show summaries of both the original figures and the means after statistical adjustment for litter effects.

On the adjusted data of the first litters (Text Table 28), none of the supplements had a significant effect on the number of young born, the number of young weaned, the number of deaths before weaning, or the total weight weaned.

With the number born, the three-factor interaction was significantly negative ($P < 0.05$), thereby showing that each supplement, in the presence of either of the others, increased the number of births, but if both other supplements were together present or absent the number of births was decreased.

The Fe x P interaction was significantly positive ($P < 0.05$) with regard to the number weaned, so that when both Fe and P were present or absent the number of young weaned was greater than when either was present alone.

The adjusted data of the second litters (Text Table 28) show that the reproductive attributes were not affected by any of the supplements except Fe, which increased both the number of young weaned ($P < 0.05$) and the total weight of young weaned ($P < 0.01$). None of the interactions reached a significant magnitude with second litters, so that of the 24 possible interactions between these three attributes (12 with each litter), two just reached significance at the 5% level. Their real existence must be considered doubtful, however, as

TEXT TABLE 27.

Exp. 2. Reproduction. Unadjusted diet-group means.

	Supplement:	Total No. born		Total No. weaned		Proportion weaned (%)		Total wt. weaned (g.)		Mean wt./ litter weaned (g.)		Mean wt./ weanling (g.)	
		Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
(a) First litters	-	76	55	66	40	85.7	72.7	480	302	60.1	50.3	7.3	7.6
	Fe	71	73	51	36	71.8	49.3	467	322	66.7	64.3	9.2	8.9
	P	56	63	50	43	89.3	68.3	465	366	58.1	61.0	9.3	8.5
	Fe + P	73	64	54	56	74.0	87.5	459	460	57.4	57.5	8.5	8.2
	Supplement:												
(b) Second litters	-	55	52	42	42	76.4	80.8	390	366	65.1	52.3	9.3	8.7
	Fe	57	74	50	65	87.7	87.8	511	579	72.9	72.4	10.2	8.9
	P	58	52	48	46	82.8	88.5	459	423	65.5	60.4	9.6	9.2
	Fe + P	72	64	61	57	84.7	89.1	610	565	76.3	80.7	10.0	9.9

Each diet-group comprised eight mated pairs.

TEXT TABLE 28.

Exp. 2. Reproduction. Adjusted diet-group means.

Supplement	No. born				No. weaned				Total wt. weaned (g.)			
	1st litters		2nd litters		1st litters		2nd litters		1st litters		2nd litters	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	9.6	7.9	8.1	7.6	8.5	6.8	7.2	6.0	60.7	50.5	67.9	52.8
Fe	8.9	9.1	8.2	9.2	6.9	5.8	7.4	8.1	60.9	47.7	75.9	72.4
P	7.0	7.9	7.2	7.5	6.6	6.2	6.0	6.4	58.7	50.7	57.3	58.4
Fe + P	9.1	8.0	9.0	8.2	7.4	7.4	7.6	8.0	61.8	60.0	76.2	78.8
Standard error of the differences between means of sets of:												
32 animals	± 0.45		± 0.62		± 0.53		± 0.65		± 4.69		± 5.51	
16 animals	± 0.54		± 0.88		± 0.75		± 0.92		± 6.63		± 7.79	
8 animals	± 0.90		± 1.24		± 1.06		± 1.30		± 9.37		±11.02	
Overall effect of:												
High $CaCO_3$	- 0.44		0.00		- 0.81		+ 0.06		- 8.28		- 3.77	
Fe	+ 0.69		+ 1.06		- 0.19		+ 1.38^{x}		+ 2.42		+16.71^{xx}	
P	- 0.88		- 0.31		- 0.06		- 0.19		+ 2.87		+ 0.45	
Interactions:												
$CaCO_3$ x Fe	0.0		+ 0.2		+ 0.3		+ 0.5		+ 0.8		+ 3.2	
$CaCO_3$ x P	+ 0.2		- 0.2		+ 0.6		+ 0.3		+ 3.4		+ 5.5	
Fe x P	+ 0.5		+ 0.1		+ 1.12^{x}		+ 0.2		+ 3.7		+ 2.9	
$CaCO_3$ x Fe x P	- 1.00^{x}		-0.7		0.0		- 0.4		+ 2.3		- 2.5	

Each diet-group comprised eight mated pairs.
For all comparisons, t is 2.0 when P is 0.05.

^{x}Significant ($P < 0.05$)
^{xx}Significant ($P < 0.01$)

TEXT TABLE 29.

Exp. 2. Reproduction. Overall means for unadjusted data.

(a) First and second litters combined

	Mean No. Born/litter		Mean No. Weaned/ litter born		Proportion weaned (%)		Mean Total wt. weaned/lit. born (g.)		Mean wt./ litter weaned (g.)		Mean wt./ weanling (g.)	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	8.2	7.6	6.8	5.9	81.8	76.6	54.4	47.7	62.6	51.3	8.1	8.1
Fe	8.0	9.2	6.3	6.3	78.9	68.7	61.1	56.3	69.8	68.4	9.7	8.9
P	7.1	7.2	6.1	5.6	86.0	77.4	57.7	49.3	61.8	60.7	9.4	8.9
Fe + P	9.1	8.0	7.2	7.1	79.3	88.3	63.8	64.1	66.9	69.1	9.3	9.1

Each diet-group comprised eight mated pairs

(b) Mean values, calculated from (a) above, for the four diet-groups containing (+) and not containing (-) each supplement

	Mean No. Born/litter		Mean No. Weaned/ litter born		Proportion weaned (%)		Mean Total wt. weaned/lit. born (g.)		Mean wt./ litter weaned (g.)		Mean wt./ weanling (g.)	
	+	-	+	-	+	-	+	-	+	-	+	-
High $CaCO_3$	8.0	8.1	6.2	6.6	77.5	81.5	54.4	59.3	62.4	65.3	8.8	9.1
Fe	8.6	7.5	6.7	6.1	77.9	81.3	61.3	52.3	68.5	59.1	9.3	8.6
P	7.8	8.3	6.5	6.3	83.3	75.9	58.7	54.9	64.6	63.0	9.2	8.7

one out of every twenty interactions might be expected to reach this level of apparent significance by chance. Certainly the three-factor interaction on 'No. born', significantly negative with first litters, was also negative with second litters - a fact which lends support to its real existence; but the Fe x P interaction on 'No. weaned', which was significantly positive with first litters, was practically zero with second litters. It seems wise to interpret these interactions with considerable reserve.

Since statistically significant results were so few, the results of the breeding trial must be considered for the most part inconclusive. It is worth while, however, examining the figures again to see whether they give any indication of an effect which might have been significant if greater numbers of animals had been used. For this purpose the unadjusted data from both first and second litters have been combined (Text Table 29(a)), and overall means struck for diet-groups containing and not containing each supplement (Text Table 29(b)). In addition, mean values for 'weight weaned per litter weaned' and 'weight per weanling' have also been calculated, and are included in the Table. These additional ratios were computed because 'total weight weaned' (per litter born) by itself has limitations as a criterion of breeding and rearing efficiency; it takes no account of the number of litters which failed to reach weaning age, nor (as we have seen above) of the number of young in those that did.

Inspection of these means confirms that differences were small. It is noteworthy, however, that the figures for the low calcium carbonate diet-groups were regularly better than those for the corresponding high calcium carbonate groups, while those for the iron groups (with one exception - 'Proportion weaned') were consistently better than those for the groups without iron. Diet-groups containing phosphate were better than those not containing it, except with the 'No. born'. These points are not really of much consequence,

however, and would scarcely be worth attention were it not for the fact tht the effects - such as they were - were generally in the expected direction.

A rather more striking point concerns the comparison between the two basic diet-groups 79 and 81 (i.e. that containing no supplement and that containing only additional calcium carbonate). With many attributes the differences between these groups was quite large, and certainly it was consistently in favour of diet 79 (except with the mean weight per weanling where the values were identical). Indeed, in many respects diet 81 appears to have been the poorest diet of all, but the presence of iron or phosphate, or both, seems usually to have offset the deleterious effects of the higher level of calcium carbonate. However, all these may well have been chance results; they cannot be accepted even as tentative conclusions, but merely as pointers to possibilities which could be further investigated in later experiments.

(iv) Other observations

(a) POST-MORTEM EXAMINATION

The results of post-mortem examinations of both dams and weanlings confirmed the findings recorded in Experiment 1. Animals from diet-groups containing iron were generally less abnormal than the others, and the influence of dietary calcium carbonate was again evident. No new features were noted, but abnormalities covered a wide range, from absent to very severe.

Examination of those young mice which perished before attaining weaning age also revealed no new features; the abnormalities were similar to those seen in the weaned animals, but were invariably very severe in degree. In some cases the stomachs were full, indicating that suckling had been maintained to the last, but in others - particularly where an entire litter died - the stomachs were empty, thereby suggesting that starvation may have been at least partly responsible for the deaths.

(b) ORGAN WEIGHTS

(My colleague Dr. Marion Richards was responsible for measuring the weights of organs, and so the detailed results will not be reported here. It will be necessary, however, to take note of her main findings in view of their relation to my own work.)

A summary of the main effects of the dietary supplements, and any significant interactions between them, is given in Text Table 30.

Body weights. These were ascertained primarily in order that the weights of organs could be considered not only in absolute terms but also in relation to body weight, but an analysis for bodyweight itself was also conducted on the figures. (The figures for litters were obtained from the four animals in each litter whose blood and organs were examined in detail, and their means therefore differ slightly from the overall mean weights per weanling, referred to above under Reproduction Data, which were averages obtained from all weanlings.)

The analysis showed that none of the supplements had a significant effect with first litters, although the Fe x P interaction, which was strongly negative ($P < 0.01$), showed that Fe increased body weight in the absence of P, but decreased it in the presence of P. With second litters high $CaCO_3$ had a negative effect ($P < 0.05$), but there were no significant dietary effects on the body weights of dams.

Heart weights. While it is true that, as a rule, the absolute weights of organs are of less interest than their relation to body weight, yet in this experiment changes in absolute heart weight reached striking levels of significance. The higher level of $CaCO_3$ markedly increased the absolute heart weights of both first and second litters ($P < 0.001$ in both cases), while Fe had the opposite effect ($P < 0.01$ in both cases). High $CaCO_3$ also increased the heart weight of dams ($P < 0.05$). Enlargement of the heart sometimes reached extraordinary proportions in weanlings from the high $CaCO_3$ groups; occasionally we found a

TEXT TABLE 30.

Exp. 2. Organ Weights. Effects of supplements, and significant interactions.

	Body weight (g.)			Heart weight (mg.)			Heart weight (mg./100 g. body wt.)			Thymus wt. (mg.)	Thymus wt. (mg/100 g body wt.)	Heart dry-matter (%)	
	1st litters	2nd litters	Dams	1st litters	2nd litters	Dams	1st litters	2nd litters	Dams	2nd litters	2nd litters	2nd litters	Dams
Standard error	±0.36	±0.40	±0.78	±2.8	±3.2	±3.9	±37.9	±66.4	±8.9	±2.2	±18.9	±0.12	±0.23
High $CaCO_3$	-0.09	-1.05^{x}	-0.77	+16.7^{xxx}	+16.2^{xxx}	+9.0^{x}	+223.3^{xxx}	+331.1^{xxx}	+44.6^{xxx}	-10.5^{xxx}	-84.4^{xxx}	-0.49^{xxx}	-0.59^{x}
Fe	+0.19	+0.48	+1.21	- 9.5^{xx}	-11.3^{xx}	+3.6	-140.0^{xxx}	-202.2^{xx}	-9.0	+7.9^{xxx}	+67.8^{xxx}	+0.46^{xxx}	+0.18
P	+0.15	+0.25	+0.51	-1.6	- 3.4	+1.1	- 50.8	- 89.3	-3.8	+2.9	+31.7	-0.06	-0.26
$CaCO_3$ x Fe													
$CaCO_3$ x P									+29.7^{xx}				
Fe x P	-1.13^{xx}			-8.8^{xx}									
$CaCO_3$ x Fe x P				+6.8^{x}			+81.6^{x}						

When P is 0.05, t is 2.02 with litters and 2.01 with dams (except for heart dry-matter when it is 2.04 with litters and 2.09 with dams)

^{x}Significant ($P < 0.05$)
^{xx}Significant ($P < 0.01$)
^{xxx}Significant ($P < 0.001$)

weanling with a heart heavier than that of its mother, although her body weight was five or six times greater. With first litters, the significant interactions showed that P had a positive effect in the absence of Fe, but that this became negative in the presence of Fe ($P < 0.01$). However, this was true only when $CaCO_3$ was present at the lower level ($P < 0.05$); at the higher level of $CaCO_3$ the effect of P was insignificant.

When the heart weights were related to body weight, the effects of the supplements were similar but even more strongly marked. High $CaCO_3$ increased the relative heart weights of first litters, second litters and dams ($P \ll 0.001$ in every case), while Fe reduced those of both first and second litters ($P < 0.001$, < 0.01 respectively). With first litters, the $CaCO_3$ effect was less in the presence of either Fe or P than in the presence or absence of both ($P < 0.05$); with the dams it was greater in the presence of P than in its absence ($P < 0.01$). It was also noted that in practically every diet-group the relative heart weights of second litters were smaller than those of first litters.

Thymus weights. The presence of the higher level of $CaCO_3$ decreased the weight of the thymus gland in second litters, whether this was considered as an absolute measurement ($P < 0.001$) or relatively to body weight ($P < 0.001$). On the other hand, the presence of Fe increased both measurements ($P < 0.001$ in both cases). There was a significant negative correlation between thymus weight and heart weight ($r = -0.436$; $P < 0.01$).

(c) HEART DRY-MATTER

Estimations made by Dr. Richards showed that the percentage of dry-matter in the hearts of second litter weanlings was significantly lowered by the higher level of $CaCO_3$ ($P < 0.001$) and increased by Fe ($P < 0.001$) (see Text Table 30). With the dams' hearts, the percentage of dry-matter was also reduced by high

FIGURE 26

Exp. 2. Litters. Correlation between haemoglobin concentration and heart weight (irrespective of diet)

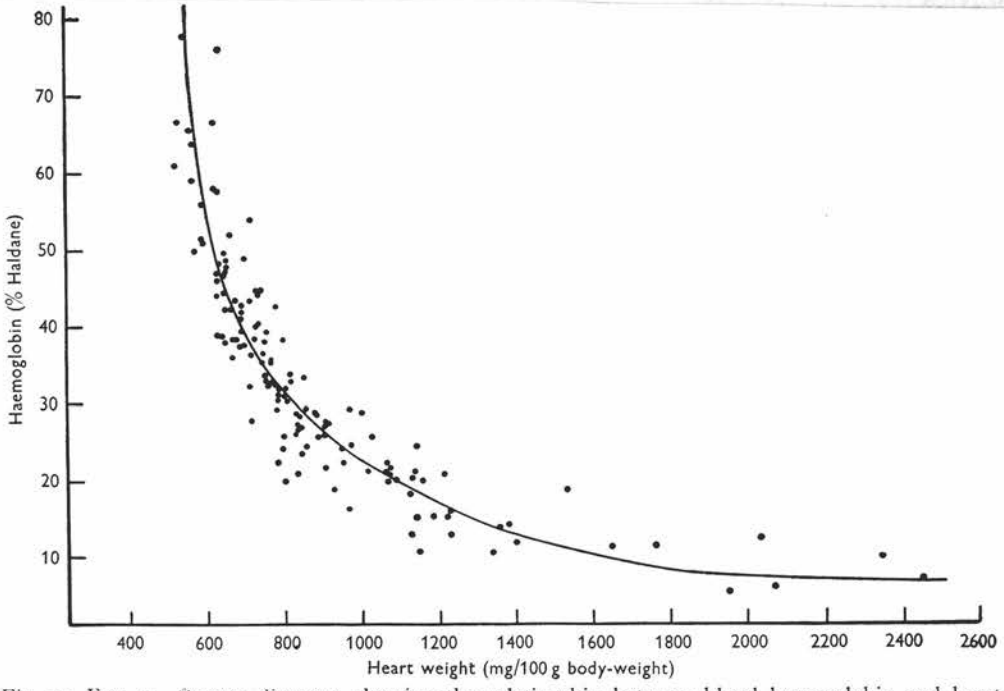

Fig. 26. Exp. 2. Scatter diagram, showing the relationship between blood haemoglobin and heart weight relative to body-weight, for 141 litters irrespective of diet. Each point represents the mean value for one litter.

$CaCO_3$ ($P < 0.05$). Examination of the results showed, however, that the increased moisture content of the hearts was by no means sufficient to account for the increased weights observed.

(d) CORRELATION BETWEEN HEART WEIGHT AND BLOOD HAEMOGLOBIN CONCENTRATION OF WEANLINGS

This correlation was investigated in the same way as in Exp. 1, and again a smooth curve resulted. It is reproduced in Fig. 26. Mr. Quenouille reported that again the relationship between the logarithms of the values was inverse and linear. The correlation coefficient r was -0.936 ± 0.010, the regression equations for the prediction of one variable from the other being:

$$\log_{10} \text{Hb.} = 5.955 - (1.533 \pm 0.049) \log_{10} \text{H.W.\%},$$

$$\text{and } \log_{10} \text{H.W.\%} = 3.780 - (0.580 \pm 0.018) \log_{10} \text{Hb.}$$

where Hb is expressed as a percentage of the Haldane standard (100% = 14.8 g. haemoglobin/100 ml. blood), and H.W.% = mg. heart weight/100 g. body weight.

(e) SERUM INORGANIC PHOSPHATE

Estimations of inorganic phosphate concentration were made on the individual serums of dams and on samples of serum pooled from all the weanlings in each litter. However, it will be more appropriate to discuss the results at a later stage (p. 211).

(f) SERUM LIPOIDS

The pale serum and hyperlipaemia noted in the blood of many weanlings in Exp. 1 was again evident in this experiment but it was never obvious in the blood of dams. From simple inspection of the plasmas or serums, there could be little doubt that the abnormality was commonest in those from the more anaemic samples of blood, and also that it was more severe in the more extreme anaemic cases.

TEXT TABLE 31.

Exp. 2. Second litters. Serum fatty acids (mg./100 ml.)

Supplement	Low $CaCO_3$	High $CaCO_3$
-	273	394
Fe	280	417
P	220	135
Fe + P	242	303

TEXT TABLE 32.

Exp. 2. Dams. Period on Diet (days)

Supplement	Low $CaCO_3$	High $CaCO_3$
-	89	88
Fe	90	85
P	83	93
Fe + P	91	84

Standard error of the differences between means of:

32 animals	± 3.3
16 animals	± 4.7
8 animals	± 6.6

Overall effects of:

High $CaCO_3$	- 0.6
Fe	- 0.8
P	- 0.2

When P is 0.05, t is 2.01.

In an attempt to confirm this observation by a quantitative procedure, I decided, after I had estimated serum inorganic phosphate, to try to utilise the remainder of the pooled serums from second litters for the chemical determination of total serum lipoids. For accurate work, however, ordinary chemical methods require the use of fairly large quantities of serum (usually 5 ml.) for each estimation, and this was of course far beyond what was available. (The yield of serum from a weanling mouse seldom exceeded 0.1 ml.) However, by pooling serum from all the litters in each diet-group, it was possible to obtain a total of 3 ml. from each, and it was therefore decided to adapt a method to the use of these smaller quantities. The principles of the method* employed, together with the modifications made to it, are described in the Technical Appendix (p. 261). These modifications, of course, diminished the accuracy of the method, but by far the most serious objection was the fact that there was insufficient serum to allow of duplicate estimations. There could thus be no way of checking the accuracy of the estimations or even of detecting gross technical errors; but as no feasible alternative was possible, I decided to make a single estimation from each diet-group, for what it might be worth.

The results of these estimations are shown in Text Table 31. In Fig. 27, the values have been plotted against the corresponding diet-group means for Hb (second litters) (see Text Table 23). These means can only approximate to the mean Hb of the bloods from which the pooled serum samples were taken, for some litters did not contribute to the pool because the supply of their serum had become exhausted. Also, those litters that did contribute serum did so to different extents, as varying quantities - in fact, as much as possible - of each blood had been collected; large litters had contributed more blood than small ones, and more serum had usually been obtained from a given quantity of anaemic

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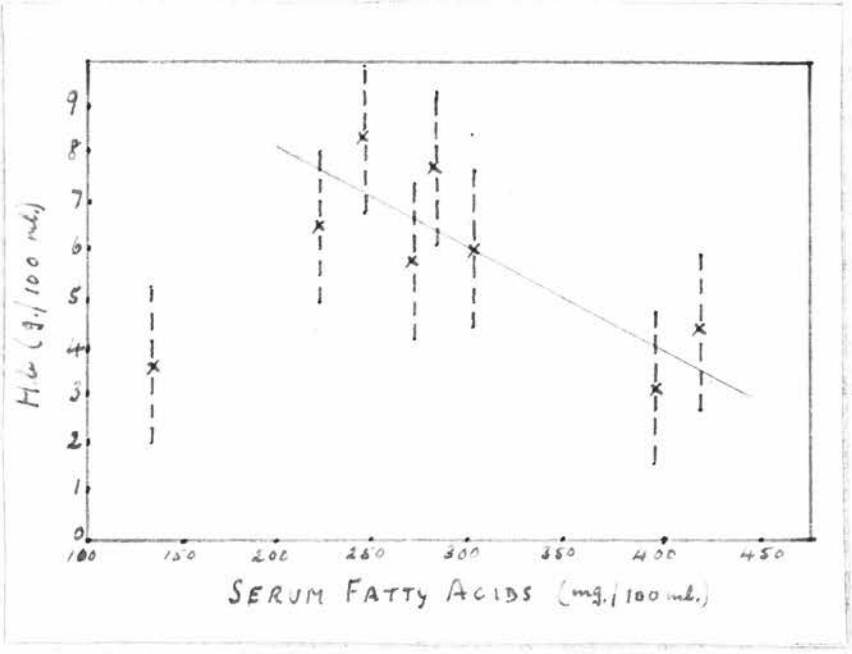
* Recommended by Dr. J. Duckworth, Head of the Department of Applied Biochemistry, Rowett Research Institute.

FIGURE 27

Exp. 2. Litters. Relationship between serum fatty acid concentration and blood haemoglobin concentration



blood than from the same quantity of more normal blood, owing to the disproportion in their cell : serum ratios. For this reason, in Fig. 27 two standard errors (equal to 1.60 g./100 ml.) have been drawn, in a broken line, on each side of the mean Hb.

Bearing in mind the opportunities for inaccuracy in their estimation, the fatty acid and Hb values show a remarkable degree of correlation. Indeed, the regression line shown in Fig. 27 passes within two standard errors of all points except one - that for the high calcium carbonate plus phosphate diet-group. As has been indicated, there is no means of assessing whether or not this exception was the result of a serious technical error in estimating fatty acids. However, it would not be surprising if any fatty acid value were much too low, as a serious and undetected loss could readily have occurred, especially at the filtration stage with an inefficient filter.

If considered by itself, the above result would, of course, be far from conclusive - an alternative interpretation, for instance, could be the existence of a Ca x P interaction, since, accepting the figures as they are, the difference between the mean effect of high $CaCO_3$ in the absence of P (+ 119) is very different from its mean effect in the presence of P (- 12). Nevertheless, when the figures are taken in conjunction with the evidence from visual inspection of the serums, it seems much more likely that one value was grossly wrong and that the result should be taken as furnishing confirmatory evidence that hyperlipaemia was most intense in the most anaemic animals.

(g) PERIOD ON DIET

Because of a considerable individual range in the intervals between the births of first and second litters, and to a less extent between first mating and birth of first litters, it occurred to me that the length of the period during which the diet had been fed might have affected the results of the

experiment to some extent.

Accordingly, I requested Mr. Quenouille to undertake a statistical analysis of "Period on Diet", the results of which are given in Text Table 32. As can be seen from this Table, however, the mean differences between diet-groups were quite insignificant, and the effects of the supplements practically zero.

(v) Summary of chief results (see also Fig. 28)

1. The higher level of calcium carbonate in the diet of breeding mice caused a lowered Hb in the blood of their first litter weanlings, associated with reductions in the R.B.C., P.C.V., M.C.H. and M.C.H.C. On the dams themselves the overall effects of this supplement were similar, and in addition it lowered the M.C.V. It also reduced the Hb of second litters, but the Hb of second litters was higher than that of first litters in every diet-group.

2. The iron supplement caused a rise in the Hb of first litter weanlings, and was associated with increases in R.B.C., P.C.V., M.C.H. and M.C.H.C. It also increased the Hb of second litters. The presence of iron also affected the dams' blood picture, causing an overall increase in Hb, P.C.V., M.C.H., M.C.H.C., and M.C.V.

3. The phosphate supplement did not significantly affect the blood picture of first litters, but it increased the Hb of second litters and dams.

4. The following interactions between treatments reached significance: first litters: Fe x P positive on M.C.V. and $CaCO_3$ x Fe x P negative on R.B.C.; dams: $CaCO_3$ x Fe positive on M.C.H.C., Fe x P negative on R.B.C., Fe x P negative on P.C.V., and $CaCO_3$ x Fe x P positive on M.C.V.

FIGURE 28

Exp. 2. Overall independent effects of dietary supplements

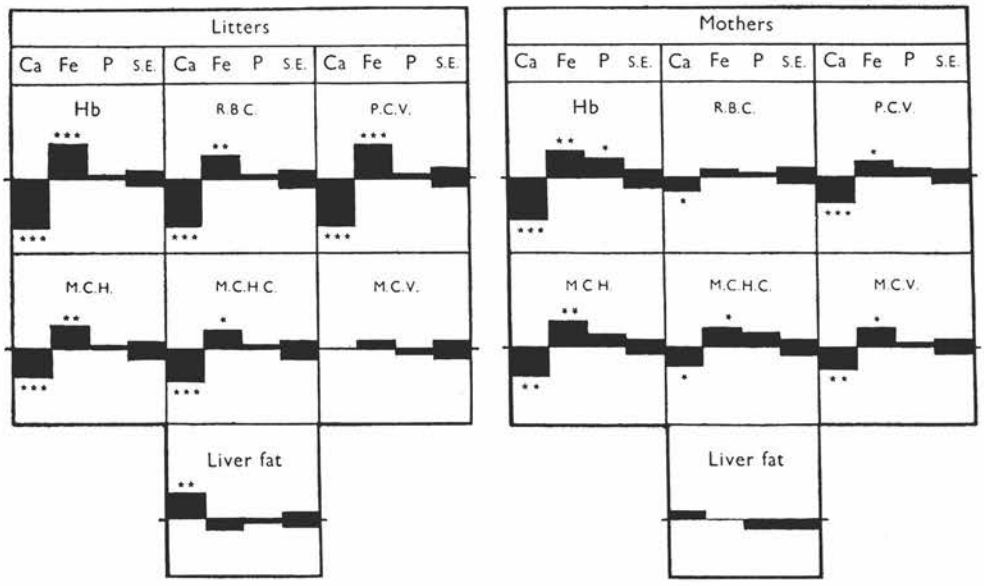


Fig. 28. Exp. 2. Overall independent effects of dietary supplements, to show the relative influence of each on various blood attributes and liver fat. The base-lines represent the overall mean measurements; positive effects (i.e. increases) above the base-line, negative effects (i.e. decreases) below the base-line. The diagram has been scaled so that the standard error is represented in each instance by the same positive and negative excursions from the base-line. Significance of effects is indicated by * ($P<0{\cdot}05$), ** ($P<0{\cdot}01$) and *** ($P<0{\cdot}001$). Ca = effect of 1·5 % supplement of calcium carbonate; Fe = effect of 10 p.p.m. supplement of iron as ferric citrate; P = effect of 0·57 % supplement of phosphorus as sodium dihydrogen phosphate; S.E. = standard error of the mean.

5. The characters of the red cells in blood films confirmed the existence of hypochromasia and microcytosis in those diet-groups where their presence would be expected from the above findings. All films, particularly those from anaemic animals, showed the presence of immature forms of red cells; even early normoblasts were often encountered in anaemic weanlings. In the most abnormal bloods, especially those of weanlings, anisocytosis and poikilocytosis were common.

6. The amount of visible fat in the livers of first litter weanlings was increased by the presence of the higher level of calcium carbonate in the mothers' diet. The presence of the iron supplement probably had the opposite effect.

7. One doe in the high calcium carbonate diet-groups failed to produce a litter, but her mate was apparently infertile. Six other does in these groups, and also one on the lower level of calcium carbonate, failed to rear their first litters; but all seven reared at least part of their second litters. The second litters of two other dams in the high calcium carbonate diet-groups, and of four in the low calcium carbonate diet-groups, succumbed before weaning.

8. With first litters, none of the supplements produced a significant overall effect on number of young born, number of young weaned, number of deaths before weaning, or total weight weaned. However, two interactions were significant, namely, $CaCO_3$ x Fe x P negative on number born, and Fe x P positive on number weaned. With second litters, the iron supplement increased number weaned and total weight weaned.

9. Post-mortem examinations confirmed the findings of Experiment 1.

10. None of the supplements had a significant effect on the body weight of first litter weanlings, but the interaction Fe x P was negative in direction. With second litters, high calcium carbonate reduced body weight.

11. The higher level of calcium carbonate increased the absolute heart weight, and also the heart weight : body weight ratio, with all classes of animal; with weanlings, the iron supplement had the reverse effect.

12. The thymus weight and the thymus weight : body weight ratio of second litters were both decreased by the high calcium carbonate supplement, and increased by the iron supplement.

13. The proportion of dry matter in the hearts both of second litter weanlings and of dams was decreased by the higher level of calcium carbonate; the iron supplement had the reverse effect with the litters.

14. There was a close correlation between the heart weight : body weight ratio and the blood haemoglobin concentration of weanlings. The relationship between the logarithms of these values was inverse and linear.

15. The blood serum of anaemic weanlings was pale and hyperlipaemic.

16. The supplements did not affect the mean period of time taken by dams to bear and rear two litters.

3. INTERPRETATION

The experiment had been designed with several objects in view. These were:

(1) to confirm certain of the results of Experiment 1.

(2) to obtain more information on the type of anaemia induced by a high level of dietary calcium carbonate.

(3) to determine whether the basal diet was improved by the addition of an iron supplement.

(4) to discover whether any or all of the effects of the calcium carbonate supplement could be countered by including an iron supplement in the diet.

(5) to investigate the effects of a phosphate supplement, and, in particular, to observe whether the effects of the calcium carbonate supplement could be offset by restoring the Ca : P ratio of the diet.

Now that we can view all the results of Experiment 2 in perspective, we are in a position to evaluate them collectively, and so to judge how far the objects of the experiment were achieved. It will be convenient to consider these objects in the above order.

(1) Experiment 1 involved supplementation of the diet with calcium carbonate, and the most striking findings concerned the effects of the supplement in the development of anaemia and in interference with reproduction. Considering the anaemia first, there can be no question that the results of Experiment 1 were fully confirmed in Experiment 2, for calcium carbonate again induced a marked anaemia in both dams and litters (see Fig. 28). With the dams, the level of blood haemoglobin was reduced by an average of about 2.3 g./100 ml. (or over 15% Haldane), this effect being more than four times as great as its standard error. The reductions were of the same order in the litters, but the effect of the supplement was more than six times its standard error with first litters, and exceeded seven times its standard error with second litters. The probability of this being a chance result is therefore less than 1 in several hundred million!

On reproductive performance, however, the effects of the higher level of calcium carbonate were very much less pronounced, and, in fact, never reached statistical significance even at the 5% level. At the same time, differences between groups were usually in favour of the groups on the lower level of calcium carbonate. It is true that the attributes were not all independent, but there was nevertheless an undoubted suggestion, more marked with first than with second litters, and more marked on rearing performance than on fertility or fecundity, that the high level of supplement was deleterious. Despite the small number of animals involved in the comparison (eight in each group), the unadjusted data, averaged over two litters, gave some indication that the presence of calcium carbonate alone was more harmful than its presence together with the other supplements. There was every indication of this in the adjusted data as well, for with each attribute the single effect of calcium carbonate alone was stronger - sometimes much stronger - than its overall effect.

Experiment 2 also provided confirmation of many of the other results, or suggestive trends, given by Experiment 1. Among these were the effects of the calcium carbonate supplement in reducing the individual body weight of weanlings, increasing the heart weight of both weanlings and dams, reducing the weight of the thymus gland in weanlings, and increasing the visible fat in weanlings' livers. Hyperlipaemia was again a feature of anaemic blood, and the close correlation previously observed in weanlings between relative heart weight and blood haemoglobin concentration was again evident.

To sum up the position, Experiment 2 had confirmed, beyond any doubt, that a high level of dietary calcium carbonate induced anaemia in both dams and litters; it also affected the weights of certain organs and

increased the quantity of fat in the livers and blood plasma of weanlings. On the question of the effects of dietary calcium carbonate on reproduction, the results were inconclusive, but I was satisfied that Experiment 2 had done nothing to overturn the results of Experiment 1, and had in many ways tended to confirm them.

(2) The detailed blood examinations, undertaken in order to classify the anaemia induced by dietary calcium carbonate, gave results which, while clear-cut in every case, were not identical in weanlings and in dams. One difference lay in the effect of the supplement on cell number (R.B.C.). With the litters, cell number was very markedly reduced, but with the dams the reduction was only moderate. Again, while with both litters and dams the quantity of haemoglobin carried by each red cell (M.C.H.) was very much reduced, the reasons for this state of affairs were different in the two classes of animal.

With the litters, the reduction in M.C.H. was accounted for wholly by the striking decrease in cell haemoglobin concentration (M.C.H.C.), with cell volume (M.C.V.) remaining unchanged. Indeed, the complete absence of effect on M.C.V. was in remarkable contrast to the very strong effects seen in all other haematological attributes. With the dams, however, the major factor concerned was a decrease in M.C.V., although a diminished M.C.H.C. was partly responsible. In other words, the anaemia proved to be hypochromic in both classes of animal, but especially in the litters; in the dams it was also microcytic, but it could not be shown to be other than normocytic in the litters. These findings confirm the major trends observed in Experiment 1.

(3) When the basal diet-group (containing no supplements except the low level

of calcium carbonate) is compared with the corresponding group containing added iron only, it can be seen that the latter held some advantage in many attributes, though not in all. The differences were largest in the haematological studies. On the other hand, it is true that in a few instances, particularly with reproductive attributes, the basal diet-group was actually slightly the better of the two, but the differences in these cases were so small, compared with the standard error, as to be quite meaningless.

It seems fair to conclude that, on the whole, the iron supplement in many respects slightly improved the basal diet, which can therefore be considered to have been slightly iron-deficient.

(4) In almost every attribute in which it was studied, the effect of the iron supplement was opposite in direction to that of the high level of calcium carbonate. This was particularly evident in the haematological attributes (see Fig. 28), but the general pattern was the same everywhere, even where the effects were not large enough for both to reach the 5% level of significance. There can be no doubt that the effects of additional calcium carbonate were countered, at least to a great extent, by the iron supplement; but there was nowhere any evidence of an interaction between them.

One aspect of the results of feeding the iron supplement merits closer examination, namely its effects on the reproductive attributes 'number weaned' and 'total weight weaned'. It is surprising to find that the overall effect of iron on 'number weaned' was significantly positive with second litters, since with first litters it was approximately zero (Text Table 28). Looking at the matter more closely, it seems likely that, with second litters, the number weaned was influenced in part at least by

the fact that the number born was higher (averaging 1.06 ± 0.62 more per litter) in diet-groups containing iron than it was in diet-groups not containing iron. This suggestion can be investigated in the following way. When the ratio 'mice weaned to mice born' is calculated, separately for diet-groups containing and not containing iron, it is found to be 0.899 : 1 and 0.842 : 1 respectively; thus, on average, the effect of the iron supplement was to increase the number of mice weaned per mouse born by 0.057. Therefore, if the number of mice born in the two groups had been equal (the overall average is 8.125), the difference in number weaned, due solely to the direct effect of the iron, would have been 0.057 x 8.125 = 0.46. When this figure is compared with the observed effect, 1.38, the large part played by the difference in the number born becomes apparent, and the effect of iron on survival from birth to weaning age is seen to be correspondingly less important.

The highly significant positive effect of the iron supplement on 'total weight weaned' is too strong to be lightly dismissed, although it, in turn, must have been largely influenced by the high number weaned in diet-groups containing iron. If the matter be more closely examined, on the same principle as above, we find that the average weights of a weanling in diet-groups containing and not containing iron are 9.75 g. and 9.23 g. respectively - a difference of 0.52 g. per weanling. If equal numbers had been weaned (the overall average is 7.09 per litter), the expected difference in weight weaned, due to the direct effects of iron, would have been 3.69 g. per litter. The observed effect, however, was 16.71 g., so that the difference in number weaned is revealed as the most important factor in its causation. Nevertheless, such a large effect on total weight weaned would not occur more often than once in a hundred times as the

result of chance alone; it follows that, although the presence of iron almost certainly contributed in more than one way to the induction of this effect, it is undeniable that the effect not only existed but was real.

(5) The effects of the phosphate supplement were everywhere relatively small. However, in two instances they reached significance at the 5% level; both concerned the highly-sensitive attribute Hb, the effects being positive with both second litters and dams. On many other attributes the effect of phosphate was also positive, but never approached significance.

The experiment showed conclusively that restoration of the Ca : P ratio did not cancel the effects of the higher level of calcium carbonate. If the phosphate had rendered the additional calcium innocuous by chemical union, then high calcium carbonate would not have exerted its effects in the presence of phosphate. In fact, however, the presence of phosphate seldom made much difference, and in the few instances where the calcium effects were perceptibly less severe in the presence than in the absence of phosphate, the effects of the phosphate were general and not confined only to the high calcium diet-groups. That this was so is shown by the $CaCO_3$ x P interaction, which, when in the direction opposite to the calcium carbonate effect, was never significant or even large. In one peculiar instance, that of the relative heart weight of the dams, this interaction turned out to be strongly positive (that is, in the same direction as the calcium carbonate effect), indicating that phosphate had actually assisted calcium carbonate to increase the relative heart weight. No other result in the experiment lends real support to the likelihood of this having been true, and no explanation is obvious. Although the probability of its having been a chance result is less than one in a

hundred, it must be remembered that the experiment involved over two hundred comparisons of this type, and that it was therefore to be expected that in at least one of them a meaningless and inexplicable difference would reach apparent significance.

On the other hand, the interaction Fe x P reached a significant magnitude on six attributes. With four of these it indicated that the presence of phosphate interfered with the effect of iron; with the remaining two - absolute heart weight of first litters and M.C.V. of first litters - the presence of phosphate appeared to increase the effect of iron. The case of the litters' M.C.V. is a special one, as will be seen later, and the interaction on heart weight is an artifact which is explained by the fact that the corresponding interaction on body weight was also markedly negative. The other four interactions pointed to mutual interference by iron and phosphate; this is not altogether surprising, since they react chemically to form insoluble products.

A mutual incompatibility between iron and phosphate was also suggested by the three-factor interaction, which on five occasions was significant and in a number of others was fairly large. On all but one of these occasions, the effect of high calcium carbonate was more intense if iron and phosphate were either not present at all or were present together, than if one or the other was present separately. Since the effects of iron certainly, and of phosphate possibly, opposed the effects of calcium carbonate, these interactions implied that when iron and phosphate were fed together their effects cancelled each other, to some extent at least. With one attribute - the dams' M.C.V. - the opposite was true. The explanation for this result is obscure, but no doubt it was related to errors in the red cell count; the influence of this factor will be discussed in the next section.

It may therefore be concluded that the effect of the phosphate supplement, while more often than not opposed to that of calcium carbonate, was always relatively small; usually it was negligible, and certainly restoration of the Ca : P ratio did not reverse the effects of the calcium carbonate supplement. In some instances, however, the presence of phosphate interfered with the effects of iron.

4. DISCUSSION

(i) Anaemia

The outstanding result of this experiment, as also of Experiment 1, was that the addition of calcium carbonate to the diet of breeding mice induced a marked anaemia in both dams and litters. The immediate cause of the anaemia appeared to be an induced deficiency of iron, this being strongly suggested by the quantitative haematological findings (with one notable exception, the unchanged M.C.V. in weanlings) together with the hypochromic, anisocytic appearance of the erythrocytes in blood films, the presence of normoblasts in the circulating blood, and the normoblastic reaction in the bone marrow. The conditions in which the anaemia occurred also support this diagnosis, for the demands of pregnancy frequently turn a marginal adequacy of iron into a frank deficiency affecting both mothers and young. Finally, there is the fact that the anaemia could be prevented by adding ferric citrate to the diet.

It might seem, then, that calcium carbonate had interfered in some way with the absorption of iron, or with its utilisation for blood formation. On the other hand, it has also been established that the basal diet was marginally deficient in iron, so that, in all groups, improvement would naturally have been expected to follow the addition to the diet of an iron supplement. It could be

argued that had the presence of calcium carbonate prohibited absolutely the utilisation of iron, then an iron supplement would have had no effect under these conditions. As this was not the case, at least it is clear that any such prohibition was not absolute. However, it could be surmised that calcium carbonate may have interfered with the availability of only a proportion of the dietary iron; but in that case the effect of the iron supplement would still have been smaller in the presence of the high level of calcium carbonate than in its absence. Again, this was not the case, for there were no important interactions between iron and calcium carbonate; that is to say, the positive effect of iron was not shown to be influenced in direction or magnitude by the level of calcium carbonate. Thus it might appear that the effects of the iron were independent of the level of calcium carbonate, and that consequently the latter must have exerted its action in some entirely different way.

However, this conclusion must be qualified by one very important consideration. There is an upper limit to the magnitude of the response possible to dosage with an iron supplement, because the degree of response is governed by the extent of the deficiency. The optimum haemoglobin concentration is also the maximum possible, and once it has been attained further supplies of available iron do not lead to further increments in the level of circulating haemoglobin. A further point in this connection is the well-recognised fact that the body's avidity for iron, and consequently the efficiency of its absorption of a given oral dose of iron, is greater in severe than in mild iron deficiency (Hahn, Bale, Lawrence and Whipple, 1938; Darby, Hahn, Kaser, Steinkamp, Densen and Book, 1947; Chodos, Ross, Adams and O'Brien, 1952). Thus if the original haemoglobin level is not very low, only a comparatively small response will be observed - in other words, improvement will be recognised only when there is room for improvement. Now, if it be assumed for the moment that the higher level of calcium carbonate did, in fact, partially interfere

with iron availability in those animals to which it had been fed, then there would be more 'room for improvement' in those animals to which it had been fed than in the controls fed only the lower level. Consequently, the magnitude of the response to the same dose of iron should have been greater in animals fed the higher level.

Thus the magnitude of the interaction could now be taken to represent a balance between two factors: (1) partial interference by calcium carbonate in the availability of iron, which would tend to limit the response given to iron by animals receiving the high level of calcium; and (2) the 'room for improvement' principle, and the reduced efficiency of iron absorption, which would tend to limit the response given by animals receiving only the low level of calcium carbonate. Depending upon which factor was predominant, the interaction would be either positive or negative: in this experiment the interaction was negligible in almost every attribute, and so it is probable that the two factors largely cancelled themselves out.

It may therefore be concluded that while the pathology of the affected animals strongly suggested that the anaemia induced by the calcium carbonate supplement was caused by iron deficiency, this point has nevertheless not been proved; at the same time, there is no evidence at variance with it.

However, iron is not the only substance whose lack will result in hypochromic anaemia - copper, for instance, is another. Preparations of iron salts, even very pure ones, frequently contain traces of copper and other metals, and it was considered possible that the effects of 'iron' noted in this experiment might really have been due, in whole or in part, to a contaminant in the ferric citrate. If so, then calcium carbonate had certainly induced a deficiency, but of something other than, or in addition to, iron; this possibility seemed worthy of further investigation.

There is another alternative explanation, but it is a purely hypothetical and extremely unlikely one. It is conceivable, however, that dietary calcium carbonate can have a specific toxic action, direct or indirect, on some aspect of haemopoiesis, this action being unrelated to a deficiency of iron. But, if this were so, it would imply not only that this hypothetical toxic action resulted in blood and marrow pathology indistinguishable from that of iron deficiency anaemia, but also that a very small quantity of iron acted, not by making good a deficiency, but as a specific antidote to the toxic action. This alternative explanation of the findings must be mentioned because it is a possibility which would fit the facts, and should therefore not be entirely overlooked; however, it is a most remote one, and there is no other evidence of any kind to support it. For the present, at any rate, it need not be seriously considered.

Assuming for the present, however, that an induced deficiency of iron was the cause of the anaemia in both the dams and their litters, an explanation must be found for the different blood pictures in these two classes of animal.

The dissimilar reductions in R.B.C. may be explained without great difficulty. Discussing the haematology of iron-deficiency anaemia, Parsons (1938) states that, at least in rats, its course is characterised by three main stages; there is at first a microcytosis with accompanying mild polycythaemia, then the erythrocytes become hypochromic, and finally erythropenia develops. This view appears to be generally accepted (Whitby and Britton, 1950; Wintrobe, 1946). If we regard our experimental animals as representing different stages in this course, then we should expect to see a very low R.B.C. in only the more severely affected animals - those which corresponded to the third, or erythropenic, stage. This was indeed the case with the dams, the least anaemic animals (those few with Hb greater than 16 g./100 ml.) being relatively polycythaemic; below this level of Hb, the R.B.C. fell only gradually in relation to Hb until the latter reached about 10g./100 ml., but below this level again R.B.C. began to fall more and more sharply. With the weanlings, however, the general level of Hb was much lower than with the dams, being less than 8 g./100 ml. in almost every

case. Consequently, the earlier stages were not represented in the weanlings, and a given fall in Hb was accompanied by a proportionately much greater reduction in R.B.C. than was the case with the majority of the dams.

It may be noted in passing that Bacharach, Cuthbertson and Thornton (1949) who studied a natural iron-deficiency anaemia in breeding rats, reported that the anaemia was less obvious in dams than in weanlings. While the results reported here agree with this finding to the extent that the variability was less in the weanlings, nevertheless the average fall in Hb was of a very similar order in both weanlings and dams. The interesting point, however, is that these workers used the red cell count as their criterion of anaemia. As the above discussion shows, this is an unjustified procedure and, indeed, it has already been criticised (Anonymous, 1950[b]). Had they instead used the blood haemoglobin concentration as their criterion, there is little doubt that the anaemia in the dams would have been more readily discernible.

The second notable difference between the blood pictures in dams and in weanlings was the relatively greater degree of hypochromasia observed in the young animals. This difference also can be readily accounted for simply by the disparity between the ranges of their Hb levels, since hypochromasia is less marked in the early than in the late stages of the condition. It is more puzzling to find that the anaemia in the young mice was not microcytic, although Hamilton, Hunt and Carroll (1933) made the same observation, which they did not attempt to explain, in piglet anaemia, and Witts (1933) has stated his belief that microcytosis is a less constant finding than hypochromasia in anaemias responsive to iron. However, from the description of the haematology of iron-deficiency anaemia given by Parsons, Hickmans and Finch (1937) and Parsons (1938), and also those of many others such as Mayerson and Laurens (1930-1), Foster (1931), Haden (1932), Price-Jones (1932), Faber, Mermod, Gleason and Watkins (1935), Guest and Brown (1936) and Josephs (1936), one would have expected microcytosis

to have been the first detectable change in the character of the red cells. Indeed, Parsons et al. (1937) suggest that the development of polycythaemia and microcytosis constitutes "the first step in the production of an iron-deficiency anaemia", and Guest and Brown (1936) state "the microcytosis appears to develop first and to be the most important sign heralding the development of the anemic condition." A similar conclusion could be reached from consideration of the principles of dyshaemopoiesis, discussed on p. 38.

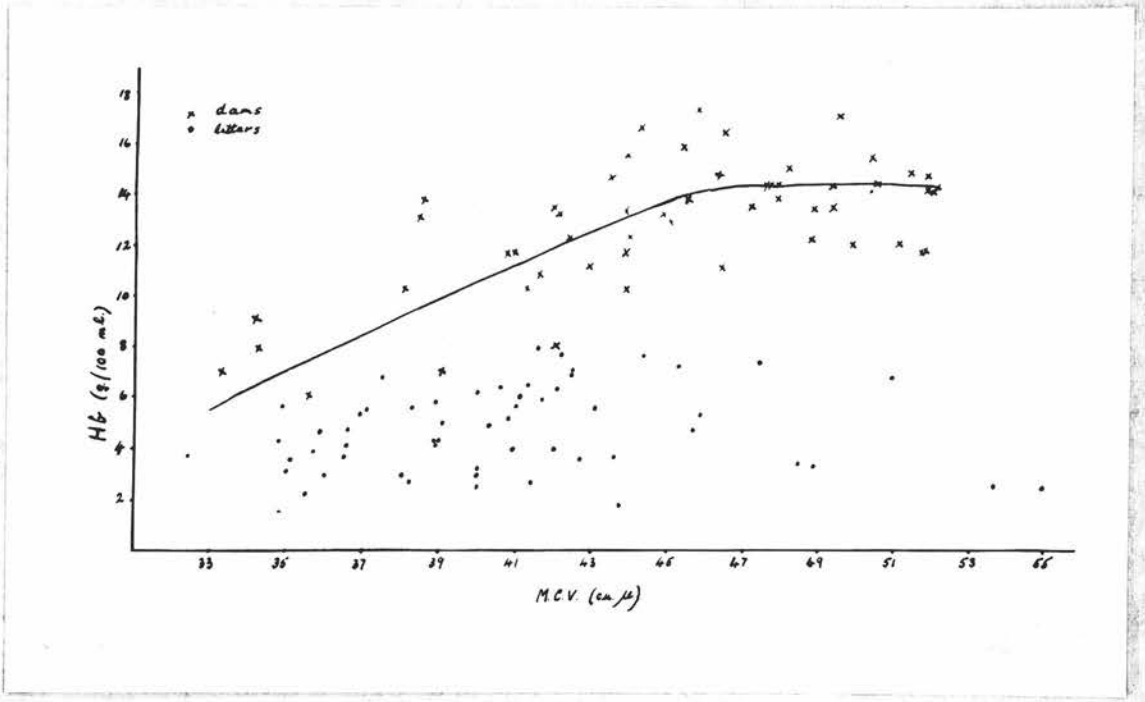
Certainly microcytosis was evident in the dams, but with the weanlings, in which the general range of M.C.V. was lower, there was little suggestion of a line of regression between Hb and M.C.V. (Fig. 29); clearly some other explanation must be found for the situation with them.

The original explanation might still hold if it could be shown that it simply did not extend far enough, and that in very severe anaemia microcytosis is for some reason or other not progressive in the later stages; the literature does not seem to contain adequate descriptions of the haematology of extreme cases of iron-deficiency anaemia. However, there is no evidence to support this conjecture. Indeed, films of the most anaemic bloods contained many obviously small cells; and if the development of microcytosis is an attempt on the part of the cell to maintain the haemoglobin concentration on the inner surface of its membrane, as is suggested by the writings of Smith, Belt, Arnold and Carrier (1924-5), Mills (1925), Emmons (1927-8) and Cruz (1941), then microcytosis would be expected to become progressively more intense as the haemoglobin level falls.

Another possibility, however, is that microcytosis in fact existed in very anaemic animals, but was masked by the anisocytosis which, as pointed out by Price-Jones (1922, 1932), is frequently seen in this condition. This suggestion seems much more plausible, and certainly anisocytosis was a well-marked feature in the films of anaemic blood. It is well recognised that

FIGURE 29

Exp. 2. Mean cell volume and haemoglobin concentration



reticulocytes are larger than mature erythrocytes, especially when blood regeneration is active (Dameshek and Schwartz, 1940), and in dogs reticulocytes have been said to have a volume about double that of adult cells (Cruz, 1941). Haden (1937) states that, in response to a strong demand, the marrow may turn out 'not quite matured' cells which are at first larger than normal. The delivery of such cells into the circulating blood would certainly account for the anisocytosis, and it may have been that a species peculiarity served to aggravate the anisocytosis in this case. Polychromatic cells, which are considerably larger than mature erythrocytes, are always numerous in the peripheral blood of normal mice, and so, in response to the stress of severe anaemia, still younger and larger cells may enter the circulation; in support of this view is the finding that the most anaemic bloods contained quite a number of early normoblasts, whereas in other species, whose blood normally contains only fully haemoglobinised red cells, such very young cells are seldom mobilised. Further, it may not be unimportant that iron, which significantly raised the weanlings' Hb, and so presumably lessened the anisocytosis, also approached statistical significance in raising their M.C.V. It is thus possible that calcium carbonate had no overall effects on the M.C.V. of weanlings because macrocytosis in the young cells balanced microcytic changes in the mature erythrocytes.

On the other hand, the estimation of M.C.V. is always subject to considerable technical error. It is calculated from a formula which reflects the errors of two observed measurements. One of these, the notoriously inaccurate R.B.C., is the denominator in the formula, so that random errors in its measurement are greatly magnified in the resultant M.C.V. This is true, of course, for all values of R.B.C.; but when the R.B.C. is small (as it was so often in the anaemic weanlings), not only will errors of the same magnitude affect the M.C.V. to a proportionately greater degree, but substantial errors in its own

measurement are more likely. Averages of many observations were taken, so that no doubt these errors cancelled themselves out to some extent, but it was clear from the full data of the experiment that variability in the recorded R.B.C. was greatest in the most anaemic animals. This means that much of the variability, and hence error, in the M.C.V. of the most anaemic animals can be ascribed to random variation in the R.B.C.

On balance, it seems likely that the usual microcytic changes did in fact overtake the mature cells, but that a parallel flooding of the peripheral blood with large immature cells preserved the mean cell volume at a roughly constant level, while the large random error inherent in low red cell counts prevented the detection of minor deviations from this mean. This explanation removes any possible objection to the conclusion that the blood picture in the most anaemic animals was of the type associated with a deficiency of iron.

One other aspect of the haematological results deserves notice, namely the significant improvement seen in second litters compared with first litters. It is unusual to find this state of affairs obtaining with iron deficiency, and indeed the reverse is usually observed (Mitchell and Schmidt, 1926; Schmidt, 1928; Kletzien and Kingdon, 1936; Alt, 1937, 1938). This is because - as has been known since the days of Bunge (1889) - infant mammals are dependent almost entirely upon their birth stores of iron during the suckling period; iron deficiency in the mother results in iron deficiency in her offspring (Scott, 1923; Schmidt, 1928; Mackay, 1931; Parsons and Hawksley, 1933; Strauss, 1933, 1934), and if the mother's stores are not built up between pregnancies, successive litters will be born with progressively smaller iron stores.

The explanation of the contrary finding in this experiment is not obvious, but may depend upon the fact that the dietary deficiency was not absolute but induced. On the assumption that the anaemia was due to iron deficiency, it could therefore be conjectured that the dams had in some way developed a tolerance

to the effects of the calcium carbonate, thus permitting more iron to be absorbed.

(ii) Fatty degeneration and hyperlipaemia

The outstanding histological feature was the fatty degeneration, especially pronounced in centrilobular areas, in the livers of weanlings from the high calcium carbonate groups. This abnormality should be considered in relation to the evidence of hyperlipaemia, since the two phenomena often have a similar origin.

FATTY LIVERS

In using the term fatty degeneration to describe the lesions seen in the livers, it is recognised that many pathologists now hold the view that the distinction between fatty degeneration and fatty infiltration, originally drawn by Virchow (1859), can no longer be accepted. The modern view, largely attributable to the work of Dible and his collaborators (Dible, 1934; Dible and Gerrard, 1938; Dible and Hay, 1940; Dible and Popjác, 1941), is that deposits of fat do not arise from the unmasking of combined fat previously contained in the cell ('phanerosis'), but have been brought to the cell from elsewhere, and that all such deposits should therefore be regarded as infiltrations. (Cameron (1952) suggests that the terms 'fatty change' or 'fatty metamorphosis' should be used to cover all forms of increase in intracellular fat). Although the evidence for this view is strong, it is by no means conclusive (see Cameron (1952) for a critical review), and Cappell (1951), while in the main supporting the modern concept, quotes further evidence which suggests that phanerosis may play at least some part in the syndrome of what was previously known as fatty degeneration.

Fatty livers can arise from several causes. Peters and van Slyke (1946) distinguish two main types. One - 'physiological fatty liver' - is only an exaggeration of a normal physiological process, additional fat having been

mobilised from the depots to meet unusual demands for the combustion of fat. The other - 'pathological fatty liver' - results from some dietary disorder, or liver injury, with the result that lipid metabolism becomes impaired and the turnover of lipids in the liver is thereby retarded.

Examples of the first type are seen in total starvation and in diabetes mellitus. In both cases carbohydrate combustion is minimal - in starvation because the carbohydrate stores are depleted, in diabetes because carbohydrate cannot be utilised. Hyperlipaemia, representing increased transfer of fats from the storage depots to the liver, and ketonaemia, resulting from the partial oxidation of these fats by the liver, are both in evidence; but there is no defect in the processes of fat metabolism. Hodge, MacLachlan, Bloor, Stoneburg, Oleson and Whitehead (1941) have given a full description of the effects of starvation on lipid metabolism in the mouse. The liver of the normal animal always contains some fat, and after the first day of starvation there is a great influx from the depots; but the whole of the carcase fat has been consumed by the end of the second day and death supervenes after four days, the liver then being quite devoid of sudanophil material. This description does not fit the situation encountered in the experimental weanling mice, although it is conceivable that semi-starvation, leading to less drastic results, might have had some influence in certain cases.

With the second type of fatty liver there is no excessive consumption of fat, and consequently there is no hyperlipaemia and no ketosis. Usually, indeed, the level of lipids in the plasma is reduced, particularly the cholesterol and phospholipid fractions (Chaikoff and Kaplan, 1934). The fat storage depots are not replenished, movement of fat being apparently blocked by the liver (Stetten and Salcedo, 1944). The causes of this type of disorder are various, but in every case the ultimate cause appears to be failure to synthesise adequate quantities of phospholipid, especially lecithin. Phospholipids, besides being

essential components of protoplasm, also appear to function in the blood plasma as transporters of neutral fat, so that a deficiency interferes with the normal flow of fat to and from the depots.

A dietary deficiency of certain essential unsaturated long-chain fatty acids, which are required for phospholipid synthesis but which cannot themselves be synthesised, will bring about a dietary fatty liver (Burr and Burr, 1930; Evans and Lepkovsky, 1932; Smedley-Maclean and Nunn, 1940; Beveridge, 1944; Beveridge and Lucas, 1945), and so, possibly, could a severe deficiency of inorganic phosphate. A number of other active compounds, the so-called lipotropic factors, are recognised. Among these is choline (Best, Ferguson and Hershey, 1933), which is usually regarded as a fraction of the vitamin B complex and is a constituent of the lecithin molecule. Its administration will prevent fatty livers in de-pancreatised dogs receiving insulin (Hershey and Soskin, 1931; Best and Hershey, 1932) or in rats on deficient diets (Best, Hershey and Huntsman, 1932; Best and Huntsman, 1932; Best, Mawson, McHenry and Ridout, 1936). Choline probably exerts its lipotropic action through its incorporation into new lecithin molecules (Welch, 1936-7, 1941; Stetten, 1941). In its absence, sufficient phospholipid cannot be formed to maintain the liver free of excess fat (Best and Ridout, 1939). Methionine, and proteins such as casein containing the methionine grouping, are also lipotropic (Channon and Wilkinson, 1935; Beeston, Channon, Loach and Wilkinson, 1936; Tucker and Eckstein, 1937; Best and Ridout, 1940) as they provide labile methyl groups necessary for the synthesis of choline (du Vigneaud, Chandler, Moyer and Keppel, 1939; Rose and Rice, 1939; Morgan, 1941; du Vigneaud, Cohn, Chandler, Schenck and Simmonds, 1941). On the other hand, substances such as cystine and guanidoacetic acid are lipogenic, because they respectively increase the demand for choline and accelerate its destruction (Beeston and Channon, 1936; Griffith, 1941; Tucker and Eckstein, 1938; Stetten, 1942; Stetten and Grail, 1942). Cholesterol is also lipogenic (Best and Ridout,

1933; Griffith and Wade, 1940; Mann, 1941), possibly by competing with phosphatides for unsaturated fatty acids.

Inositol, also a fraction of the vitamin B complex, is another compound which must be classed as a lipotropic factor, but its action is not similar to that of choline (Gavin, Patterson and McHenry, 1943; Beveridge, 1944; Best, Lucas, Patterson and Ridout, 1946; Handler, 1946), although, like choline, it probably acts by assisting in the formation of phospholipids (McHenry and Patterson, 1944). Inositol is a constituent of certain essential phospholipids (Anderson and Roberts, 1930; Folch and Woolley, 1942), and its deficiency can lead to fatty livers (Gavin and McHenry, 1941a; Forbes 1943-4; Abels, Kupel, Pack and Rhoads, 1943-44). A relative insufficiency of inositol can be induced by an excess of biotin (Gavin and McHenry, 1941a, b) or by a deficiency of pyridoxin (Engel, 1942) or pantothenic acid (Scudi and Hamlin, 1942), all of which appear to increase the requirement for inositol.

On the other hand, some other members of the vitamin B complex besides biotin are lipogenic. Thiamin and riboflavin both promote the development of fatty livers, but probably only because they stimulate metabolism (McHenry and Gavin, 1938; Gavin and McHenry, 1940). Large amounts of nicotinic acid have a similar effect, probably through competition with choline for methyl groups (Handler and Dann, 1942).

The fatty liver resulting from damage to the parenchyma of this organ is classed by Peters and van Slyke (1946) in the same group as the dietary fatty livers just referred to. In this case the necessary materials for the formation of phospholipid are available, but the machinery for their utilisation is impaired, with the result that fat entering the liver from the depots merely accumulates. Cameron (1952) states that "perhaps it is no exaggeration to say that anything that damages cells will, under the proper conditions, produce fatty change." It is well known that protoplasmic poisons such as phosphorus,

chloroform and carbon tetrachloride induce severe fatty lesions in the liver, as do some bacterial toxins (Cappell, 1951). Fatty change commonly follows cloudy swelling, and may even be associated with areas of necrosis. The fatty liver of chronic alcoholism in the human subject may also arise from liver damage, though in the opinion of Himsworth (1947) a more important reason is the impairment of appetite and deficient intake of lipotropic factors.

At this stage, it can be remarked that there is no obvious reason why any of the factors so far mentioned - with a few exceptions - could not have been responsible for the fatty livers observed in my experimental weanling mice. A deficiency of inorganic phosphate, however, could not have been the cause, as the supplement given in Experiment 2 did not affect the quantity of fat in the liver. Nor could an excess of a lipogenic factor or an exogenous protoplasmic poison have been concerned, since the lesions followed merely the addition of calcium carbonate to the diet. It is also very difficult to imagine that a deficiency of an essential fatty acid could have been induced, as the dams' diet was rich in milk which, in turn, is rich in essential fats.

However, three other types of fatty liver must be mentioned. One is the liver of 'pathological adiposity' - the fatty infiltration of Virchow - associated usually with simple obesity, although it is seen in other circumstances such as recovery from severe illness, especially typhoid fever in man (Cappell, 1951). It appears to arise merely as a means of storing fat, which accumulates around the portal tracts, and thus it does not conform to the requirements of either of the groups of fatty livers distinguished by Peters and van Slyke (1946). In my mice, however, the affected livers were not of this type, as the sites of the fatty lesions were centrilobular and not periportal.

Second is the fatty liver associated with anoxia, whether ischaemic or anaemic in origin. This again defies classification in either of the two groups, but it is nevertheless well recognised (Boyd, 1938; Cappell, 1951). The

mechanism of its development is not certainly known, but Dible (1951) explains it by saying "we assume that fat comes to the cell in the ordinary way, but owing to lack of oxygen its utilisation is slowed, and so it accumulates in the cell's protoplasm." The remaining type of fatty liver is associated with certain endocrine disturbances. The 'anoxic' and 'endocrine' livers merit discussion in more detail.

Fatty changes in tissues such as heart, liver and kidney may follow partial anoxia due to circulatory failure or embolism. They are also frequently seen in anaemia, particularly pernicious anaemia in the human subject, and appear to be due solely to anaemic anoxaemia (Cappell, 1951). As will be seen a little later, hyperlipaemia may also be associated with anaemia. Such changes have also been produced experimentally. According to Cameron (1952), as early as 1870 and 1884 respectively, first Tschudnowsky and then Albitzky described the production of fatty livers in animals by repeated bleedings or by exposure to low oxygen tensions, though Perl (1874) was less successful. More recently, Campbell (1928) kept animals at a very low oxygen tension (equivalent to that on the summit of Mount Everest) for periods of 7 to 53 days. Nearly half of his animals died, with extreme congestion of the liver, lungs and other organs. All animals showed marked atrophy and even necrosis of the liver cells, and occasionally oedema was evident in some organs. The capillaries were often dilated, especially in the livers, and in guinea-pigs and rabbits the hearts and livers also showed extreme fatty degeneration; but this change was only slightly, if at all, evident in rats and mice. Rich (1930) stated that central atrophy of the liver lobules, simulating chronic venous congestion, is the most marked feature at autopsy in well-nourished but anaemic human patients, and he described similar findings in dogs and guinea-pigs exposed to low oxygen tension and in dogs subjected to simple haemorrhage. While he agreed that fat is often seen in the liver cells in cases of anaemia, he concluded that "anoxemia specifically produces atrophy of the

hepatic epithelial cells" and that "the cells about the efferent vein, in contrast to those in the periportal region, are definitely more sensitive to the effects of a deficient oxygen supply." Rosin (1928) reported similar findings with guinea-pigs and rabbits at low barometric pressure or under reduced oxygen tension, although he also observed very severe fatty changes in the centre of the lobules; but mice and rats were little affected, and thyroidectomised rats, particularly, showed no pathological evidence of oxygen lack. Himsworth (1947) has cited other reports of centrilobular liver necrosis following exposure to atmospheres deficient in oxygen.

While these accounts suggest that centrilobular atrophy or even necrosis is a more usual finding than fatty change in anoxaemic states, especially in mice, there was no evidence of these lesions in my anoxaemic mice. In some cases the sinusoids were distended, but the only constant feature was fatty metamorphosis. However, it must be remembered that although many of these animals were severely anaemic, the degree of anoxaemia would hardly be as extreme as in many of the experiments described above. Further, McMichael's (1937) work leads to the conclusion that the important factor in the causation of anoxic liver damage is not so much the total amount of circulating oxygen per unit volume of blood, as the tension at which the oxygen is held. Himsworth (1947) has adduced further evidence to support this view. In that case, the degree of anoxia in my mice might not have been serious enough to cause atrophy or necrosis in the liver cells, and yet might have been severe enough to result in the milder lesion of fatty metamorphosis. In this connection, it is very significant that Schmidt (1928), working with mice on iron-deficient diets, reported histological findings very similar to those recorded here, and also observed the associated hyperlipaemia. At the same time, he frequently found fatty deposits in the myocardium, pulmonary alveolar cells, and glomeruli of the kidneys, and also centres of calcification in many of the enlarged hearts. Such lesions were not seen in

the present experiments, apart from one instance of calcification in the myocardium; but in Schmidt's animals the anaemia was of longer duration.

It seems very likely, then, that the fatty change could have been caused by local anoxia in the liver, and might therefore have been simply a reflection of the severe anaemia. This view is supported by the centrilobular distribution of the fat (areas furthest from the blood supply being most affected) and by the degenerative changes in other highly specialised and active tissues, such as heart muscle and kidney tubules, which would probably have been affected if the oxygen-carrying capacity of the blood had been reduced. It was notable, however, that these changes did not proceed to fatty degeneration.

Although the iron supplement did not significantly reduce the amount of fat in the livers, as judged by the rather crude method of microscopical assessment, there was nevertheless a suggestion of such an effect. Yet it certainly raised the blood haemoglobin concentration, and a comparison of these values and those for liver fat, in individual weanlings, yields further supporting evidence for the suggestion that the liver changes were the result of anoxia. Using the raw data, of course, it would be expected that the correlation would be strong, since it has been shown that diet affected both variables. The effect of all supplements was therefore eliminated, as were any litter differences, before the degree of correlation was measured. When this had been done, the correlation still proved to be negative, and significantly so at the 2% level (i.e. $P < 0.02$). The value of r was -0.373. This result suggests that the amount of liver fat was determined by the blood haemoglobin concentration; but, however unlikely the alternatives may seem, the correlation must be interpreted as merely suggesting and not proving. For instance, the correlation would also be consistent with the reverse situation - that liver fat affected haemoglobin concentration, while the existence of some third factor, independent of diet or litter but exerting its influence on both liver fat and haemoglobin level, cannot be ruled out.

The endocrine organs which have been recorded as influencing liver fat are the pancreas, the hypophysis, the ovary and the thyroid gland. Deficiency of insulin, the hormone of the pancreas, causes diabetes mellitus; the fatty liver associated with this disease is of the 'physiological type' and has already been mentioned. The hypophysis secretes a diabetogenic hormone, and extracts of the gland can induce a fatty liver (Best and Campbell, 1936); but this effect appears to be only a special case of carbohydrate starvation. On the other hand, hypophysectomy per se appears to have little or no effect on the liver lipids (Chaikoff, Gibbs, Holtom and Reichert, 1936). Oestrogens can induce fatty changes in the liver. Castrodale, Bierbaum, Helwig and Macbryde (1941) also noted central necrosis in the livers of dogs of both sexes which had received large doses, while Stamler, Miller, Akman, Silber, Bolene and Katz (1950) are among those who have remarked hepatic lipidosis in chickens treated with oestrogenic substances. Both hypophyseal and oestrogenic hormones will be further discussed in relation to hyperlipaemia.

The thyroid gland also exerts an important influence on liver lipids. The thyroid hormone appears to have a lipotropic effect, its deficiency leading to fatty livers in dogs (Entenman, Chaikoff and Reichert, 1948) and rats (Handler, 1947, 1948). The latter author found that the quantities of both cholesterol and neutral fat in the liver were increased by thiouracil administration, but that feeding thyroid material in the diet reversed these changes, whether the animals were choline-deficient or not. Abelin and Klingler (1948) reported that rats fed active thyroid material first lost fat from the reserves in the subcutaneous and muscular regions, and later from the liver. They regard thyroxine as the chief hormone involved in the breakdown of fat, exerting its influence chiefly through increased oxidation. A clear description of the histological features of the fatty liver seen in hypothyroidism is given by Chaikoff, Entenman, Rinehart and Reichert (1943). They describe pericentral

fatty change, together with oedema, cell infiltration, and early fibrosis around the central and sublobular veins, the portal tract remaining normal. The distribution of the fatty changes and oedema is thus similar to that seen in my experimental mice, although cell infiltration and early fibrosis were not seen. The evidence certainly suggests that hypothyroidism could have been at least partly responsible for the liver lesions in these animals.

Conclusions. The co-existence of fatty liver and hyperlipaemia in the experimental weanling mice from the high calcium carbonate diet-groups suggests that the liver lesions were either of the 'physiological' type (starvation or diabetes mellitus), or were caused by anaemic anoxia or an endocrine disturbance, especially thyroid deficiency. However, a superimposed induced dietary deficiency of a lipotropic agent cannot be ruled out; these agents include choline, methionine and inositol, but the lipotropic action of methionine is not distinct from that of choline, as it depends entirely on the fact that methionine assists in the synthesis of choline.

HYPERLIPAEMIA

By hyperlipaemia (sometimes described simply as 'lipaemia') is meant a higher than normal level of fat and lipoid in the blood plasma. Strictly speaking, the term refers only to the neutral fat fraction of the blood lipids - the portion responsible for lactescence ('milkiness' or 'creaminess') in plasma; but it is often used with reference to total plasma lipids, which include neutral fat and fatty acids, cholesterol and its esters, and phospholipids.

A transitory elevation of the plasma lipid level - so-called "physiological", "alimentary" or "post-prandial" hyperlipaemia - is well known to follow a meal rich in fat (Bloor, 1914, 1915; Man and Gildea, 1932-3; Rubin, 1939), but obesity per se is not associated with changes in the level

of circulating lipids (Bruger and Poindexter, 1934; Peters and Man, 1943a). However, hyperlipaemia has been recorded in association with a considerable variety of pathological states.

Bloor (1921) considered the cause of pathological hyperlipaemia to be "a disturbance of the balance between inflow and outflow of fat in the blood", following either diminished utilisation of fat by the liver or increased withdrawal of fat from the body storage depots. Such conditions are sometimes referred to as "transportation hyperlipaemia", and are all pathological. Milbradt (1930) believed that the concentration of lipids in the plasma is determined by the amount of fat metabolised, but, as has been clearly shown by Flock, Corwin and Bollman (1938), there are other factors also concerned - "more subtle circumstances of its metabolism which are as yet ill-defined" (Peters and van Slyke, 1946).

A condition known as "essential" or "idiopathic" hyperlipaemia is found in the human subject; its cause is unknown but it appears to be a familial disorder (Bernstein, Williams, Hummel, Shepherd and Erickson, 1939; Chapman and Kinney, 1941; Movitt, Gerstl, Sherwood and Epstein, 1951). Hyperlipaemia has also been recorded in connection with von Gierke's disease (Thannhauser, 1940), and with certain lipoidoses, such as Niemann-Pick disease (Pick, 1933), Tay-Sachs disease, the xanthomatoses, and occasionally Gaucher's disease (Peters and van Slyke, 1946). Such conditions can, of course, be immediately excluded in considering possible causes of the hyperlipaemia seen in the weanling mice from mothers on diets high in calcium carbonate. In addition, certain other recorded causes of transportation hyperlipaemia could obviously have played no part in the aetiology of the condition - such, for instance, as obstructive jaundice (Epstein, 1932; Epstein and Greenspan, 1936; Man, Kartin, Durlacher and Peters, 1945), renal disease (Peters and Man, 1943c) especially lipid nephrosis and the nephrotic stage of glomerulo-nephritis

(Bloor, 1916, 1917; Lichtenstein and Epstein, 1931; Thomas, Rosenblum, Lander and Fisher, 1951), chronic alcoholism (Cappell, 1951), severe exercise (Murlin and Riche, 1916; Patterson, 1927; Stewart, Gaddie and Dunlop, 1935), excessive administration of vitamin A (Josephs, 1939, 1942, 1945) and certain forms of poisoning (phosphorus, chloroform, carbon tetrachloride, phlorizin etc. (Lehnherr, 1935; Gray, 1930). Nor could the supplement have had a direct action on fat absorption in the dams - with a consequent passing on of additional fat to the young via the milk - as it has been shown that dietary calcium carbonate has no significant effect upon faecal excretion or retention of lipids (Schulz and Thomas, 1950).

Among other conditions in which hyperlipaemia is known to occur are pregnancy and lactation, ether anaesthesia, avitaminosis B, starvation and malnutrition, certain forms of anaemia, and certain disorders of the endocrine system, notably diabetes mellitus, hypothyroidism, anterior pituitary dysfunction, and hyperoestrogenism. These conditions all warrant - and will now be made the subject of - closer examination, since there are no obvious reasons why they could not have been involved in the mice.

Pregnancy and lactation. Serum lipids, including fatty acids, are high in pregnancy in the human subject (Tyler and Underhill, 1925; Boyd, 1934), but the cause of this remains unknown. In the pregnant rabbit, however, the level of blood lipoids is apparently reduced (Baumann and Holly, 1925-6). In some species, including cattle, serum lipids remain high during lactation (Maynard, Harrison and McCoy, 1931; Schaible, 1932), but in women they may return to normal after delivery (Gardner and Gainsborough, 1929; Boyd, 1935). Tyler and Underhill (1925), on the other hand, found that blood lipids remained high during the first two weeks of the puerperium. Since the cause of these changes is unknown, and also because of possible species differences,

it is very difficult to decide whether hyperlipaemia in a pregnant or lactating mouse doe could affect the plasma lipids of her young; there is, however, no evidence that it can do so. The plasma lipid levels of the dams were not determined, but their serum exhibited no obvious hyperlipaemia. A physiological hyperlipaemia in the young, following a meal of very fatty milk, is a possibility; but it is a rather remote one as the normal milk of the mouse already contains a high proportion of fat, and weanlings from all groups were removed from their mothers at the same time. It seems likely that the cause of the condition must be looked for elsewhere.

Ether anaesthesia. That ether anaesthesia causes hyperlipaemia has been shown by Bloor (1914, ~~1916~~), Mahler (1926) and Hospers (1933). While it is true that the hyperlipaemic weanling mice were subjected to this treatment before blood samples were withdrawn, it is also the case that the control mice from the low calcium carbonate groups were similarly anaesthetised. Ether anaesthesia may therefore have been responsible for a rise in the serum lipid level in all groups, but it could not have accounted for the differences between groups.

Avitaminosis B. As has been mentioned, certain components of the vitamin B complex, notably lipotropic factors such as choline and inositol, are intimately linked with fat metabolism; but hyperlipaemia does not seem often to have been reported in connection with deficiencies of any individual fractions of this vitamin. Indeed, the dietary type of fatty liver is usually associated with hypolipaemia. Sure and Smith (1931, 1932), however, reported that deficiency of the vitamin B complex resulted in a marked hyperlipaemia in the rat whether lactating, nursing or weaned, the fatty acid fractions being particularly affected. They also found that the liver glycogen reserves were depleted. These effects were independent of the food intake, and were particularly

interesting in view of earlier work by the same group of investigators (Sure, Kik and Walker, 1929; Sure and Smith, 1929; Sure and Walker, 1932) which showed that deficiency of the vitamin B complex in the rat caused a disturbance in haematopoietic function.

In Experiment 1, however, no obvious differences in the severity of the hyperlipaemia had been noticed between the rows, which varied in vitamin B content. It is therefore most unlikely that avitaminosis B was responsible for the hyperlipaemia.

Starvation and malnutrition. The effects of malnutrition, fasting and total starvation on the plasma lipids are apparently very variable, particularly in the earlier stages of these conditions. Bloor (1921), for instance, reported that fasting induced hyperlipaemia in dogs, but Entenman, Changus, Gibbs and Chaikoff (1940) were unable to confirm this. Kartin, Man, Winkler and Peters (1944) also noted no elevation of serum lipids in dogs, but observed that hypolipaemia followed fasting in monkeys and in man, although the neutral fat fraction was little affected. Man and Gildea (1936) have reported that serum fatty acids were below normal in 16 out of 31 emaciated human patients, Hodges, Sperry and Andersen (1943) found serum cholesterol low in 78 patients with wasting diseases, and Sure, Kik and Church (1933) found a reduction of serum fatty acids in starved rats. Most of these observers also noted alterations in the ratios of cholesterol, phospholipid and neutral fat. However, when the diet is deficient in carbohydrate but otherwise adequate, hyperlipaemia seems to follow regularly, particularly in children (Underhill and Baumann, 1916; Tolstoi, 1929; McQuarrie, Husted and Bloor, 1933).

These results may be explained by the fact that in starvation, lipids must be mobilised from the depots in unusually large amount to provide for energy production, and serum lipids would therefore be expected to rise and

remain high; but when the depots become exhausted, the level must fall again (Peters and van Slyke, 1946). These authors consider that the hyperlipaemia of starvation is related to the ketosis that attends this state, and appears to involve mainly cholesterol and phospholipids, although the neutral fat fraction, if examined "at the proper interval in the earlier stages of starvation", may be found to be elevated. There also appear to be species differences; starvation hyperlipaemia is more easily induced in those animals (such as rabbits and other vegetarians) whose plasma lipids are normally low, than in others (such as dogs and other carnivors) which are accustomed to a high plasma lipid level (see also p.110).

As has already been noted, starvation may have been at least partly responsible for some of the deaths in the sucking mice, though not for all of them. It is also possible that some of the weanlings, although still surviving, had been unable to obtain sufficient nourishment from their mothers, and were thus in a state of semi-starvation; and it is most likely that the majority of such animals were in the high calcium carbonate groups, since these groups had also seen the greater number of deaths. However, by no means all of the animals from these groups were in this condition. They had been taken away from their dams only a few minutes before being sacrificed, and their stomachs usually contained curdled milk and sometimes solid food from their mothers' rations; moreover, as has been previously noted, their carcases were frequently covered with layers of fat. Starvation or semi-starvation may have exacerbated the hyperlipaemia in some instances, but it could hardly have been entirely or even mainly responsible.

Anaemias. The fact that anaemia co-existed with hyperlipaemia in the experimental mice necessitates detailed consideration of their possible relationship. Among the earliest experimenters in this field were Boggs and Morris (1909), who made the important observation that, if rabbits were

exsanguinated daily, hyperlipaemia developed when the erythrocyte count fell to 2 millions per cu. mm. The condition was so pronounced that in some instances the blood lipids were increased to twenty times the normal level. They noted, particularly, an increase in the lecithin fraction, and, from the fact that much of the fatty material neither stained with fat stains nor was extractable with fat solvents, they deduced that the condition was dissimilar to the hyperlipaemia of diabetes. They also observed a high level of serum calcium, and found that the fat could be extracted if the calcium were removed with oxalate; this suggested to them that the fatty material in the serum was held bound to calcium. A further observation was that the serum fatty acids were highly unsaturated. Sakai (1914) repeated and confirmed the main part of this work, and reported also an increase in plasma cholesterol. Horiuchi (1920b) also confirmed these findings, and observed that the condition occurred at higher erythrocyte counts if the animals had been fed a diet rich in fat. He noted, further, that hyperlipaemia developed after the administration of haemolytic agents; the increase was mainly in the neutral fat portion, but the cholesterol and lecithin levels were also raised. Similar findings have been reported in rabbits by many other investigators, including Milne (1912-13), Ellermann and Meulengracht (1917), Fishberg and Fishberg (1927-8), Johansen (1930), Kumpf (1932), Chamberlain and Corlett (1932) and Stewart (1942). Feigl (1921) reported that an exsanguination hyperlipaemia could be induced in the guinea-pig, and that all fractions of the serum lipids were affected. He found similar changes in the blood of human beings following extensive haemorrhage. In rabbits, Starup (1934b) induced exsanguination hyperlipaemia even after severance of the spinal medulla.

On the other hand, Terroine (1920) was unable to demonstrate such a reaction in the dog, a finding confirmed by Bloor (1921), who noted, however, that if a fatty meal were then fed, a slight transient hyperlipaemia became

demonstrable. Bodansky (1925) was unable to alter the plasma fatty acids of dogs by means of an experimental haemolytic anaemia, although the plasma cholesterol level fell; but in a similar condition Dubin (1918) noted an increase in total fats although the lecithin and cholesterol levels were both lower.

In pernicious anaemia in man, Csonka (1916) found no change in total blood lipids, although there was a fall in the cholesterol level. In both pernicious anaemia and so-called secondary anaemia in man, Bloor and McPherson (1917) observed no change in plasma lipids until the erythrocyte count fell to about half its normal value, but thereafter neutral fat increased and cholesterol and phospholipid values fell. In pernicious anaemia, Williams, Erickson, Bernstein, Hummel and Macy (1937) found an increase in plasma neutral fat with a concomitant deficiency of cholesterol esters and phospholipid, but these returned to normal levels after successful therapy; Williams, Erickson, Bernstein and Macy (1940) showed that all the phospholipids were affected in the hypophospholipidaemia. Miller (1930) also observed a rise in lipoids following treatment of pernicious anaemia with active liver extract, but was unable to demonstrate any change in fatty acid levels at any stage.

Similarly, Erben (1902) observed an increase in serum fat, but a decrease in serum cholesterol and lecithin, in iron-deficiency anaemia. Erickson, Williams, Hummel, Lee and Macy (1937) also noted an elevation in the level of plasma neutral fats in hypochromic anaemia, and also in haemolytic anaemias of childhood. Others who have noted hypocholesterolaemia in association with anaemia, both pernicious and iron-deficient, include Denis (1917), Bloor and Knudson (1917), Pacini (1918), Feigl (1919), Kipp (1920), MacAdam and Shiskin (1922-3), Köhn (1925), Oser and Karr (1925), Beck (1928), Muller and Heath (1933), and Hodges, Sperry and Andersen (1943). More recently, however, again in man,

Froehlich (1950) found a decrease in all fractions of the blood lipids in iron-deficiency anaemia, and also in anaemia associated with chronic haemolytic jaundice. The same investigator (Froehlich, 1935, 1950) reported no change in the total blood lipids in pernicious anaemia, but deducted that, since the cholesterol and phospholipid levels were low, there must have been a compensatory increase in the triglycerine fraction.

It can be inferred, then, that anaemia *per se* has no constant effect on the total plasma lipids in man or in the dog, although hypocholesterolaemia and hypophospholipidaemia have been regularly observed in all types of anaemia. Consequently, it seems highly probable that plasma fatty acids must be increased in most cases, and Csonka (1918) has shown that these acids are abnormally unsaturated in naturally-occurring anaemias. But apparently there are important species differences, for the picture is different in some other animals, particularly the rabbit. The fact that exsanguination hyperlipaemia may easily be induced in the rabbit and guinea-pig, but not the dog, and that alimentary hyperlipaemia is difficult to induce in the rabbit but not in the dog (Sakai, 1914; Bloor, 1932) led Bloor (1932) to point out that the dietary habits of these animals are quite different (see also p.107). He therefore postulated that the differences were accountable to different rates of fat metabolism. The dog, accustomed to ingest large quantities of fat, absorbs it into the plasma and utilises it quickly for energy production. The rabbit, on the other hand, is herbivorous in habit, depends mainly on carbohydrate as a source of energy, and possesses no mechanism to deal promptly with large amounts of lipid. It does not absorb fat readily, and if the plasma is flooded with such material, in response to haemorrhage, this animal is unable to oxidise it rapidly (Horiuchi, 1920b). MacLachlan (1939) came to the same conclusion. Presumably man falls into the same category as the dog, while the mouse, because of its predominantly vegetarian habits, would be expected to behave

similarly to the rabbit in this respect.

Certain other variables suggest themselves as important. For instance, Muller and Heath (1933) noted that in acute haemorrhage in the human subject the level of blood lipoids was unchanged or high, whereas in chronic haemorrhage it tended to be low. Again, Boyd and Stevenson (1937)[8] observed that in rabbits subjected to a single massive haemorrhage the plasma lipids were first lowered in concentration. This decrease affected phospholipids and cholesterol, but not neutral fat. Within 24 hours the lipoid values were restored, while after 48 hours a great increase in neutral fat had taken place and hyperlipaemia was impending. Chamberlain and Corlett (1932) made similar observations. They found with rabbits that a single large haemorrhage, or repeated small haemorrhages, led to a fall rather than a rise in plasma cholesterol (which they attributed to blood dilution), and that a secondary rise did not usually follow; whereas repeated large bleedings invariably produced a massive secondary increase in cholesterol, as part of a general hyperlipaemia. They also pointed out that this hyperlipaemic response takes place not only in experimental exsanguination, but in all forms of anaemia. MacLachlan (1939) concluded from his work with rabbits subjected to short periods of reduced atmospheric pressure that anoxaemia results in a movement of neutral fat from the plasma to the liver, but that if the stimulus is maintained there follows a compensatory mobilisation of depot fat.

Consideration of all the evidence leads to the conclusion that severe or prolonged anaemia is always followed by a mobilisation of neutral fat from the storage depots, but that at any given moment the value of the total lipid content of the plasma will depend on whether the increase in neutral fat does or does not balance a possible decrease in the other fractions, these being determined in turn by the balance between rates of mobilisation and rates of removal.

Opinions have also differed on the mechanism by which hyperlipaemia develops in severe anaemia. Horiuchi (1920a) showed that normal rabbits, dogs and human beings all have a species and individual constancy in the lipid content of their blood; at least in the dog, this individual constancy is quite remarkable (Terroine, 1920). It is obvious, then, that any departure from this normal must be the result either of increased mobilisation of lipids into the blood, or of their diminished destruction or excretion. Bloor (1921) recognised this, and suggested that the immediate cause of haemorrhagic hyperlipaemia was an abnormally large inflow of fat. Sakai (1914) and Milbradt (1930) also attributed the condition to a mobilisation of tissue fats, believing this to be the body's reaction to a loss of blood protein; this suggestion had been advanced originally by Boggs and Morris (1909), and it received strong support from Fishberg (1929). She noted, following the demonstration (Fishberg and Fishberg, 1927-8) that exsanguinated rabbits develop hypoproteinaemia as well as hyperlipaemia, that the rise in plasma lipids was more closely correlated with the fall in serum proteins than with the diminution of red blood cells, and concluded that the lipids had been mobilised in an effort to overcome a decrease in "colloid osmotic pressure". Johansen (1930) obtained similar results, but would not agree with Fishberg's conclusion, remarking that "one can only say that lipemia is coincident with low colloid osmotic pressure". This view is supported by the subsequent work of Leiter (1931) and Shelburne and Egloff (1931), who were able to induce hypoproteinaemia and oedema in dogs without the development of hyperlipaemia; but of course the species difference must also be remembered. Another explanation was put forward by Sakai (1914) and Horiuchi (1920b), who suggested that loss of blood lipase was responsible; but as Bloor (1921) has pointed out, "this explanation is not convincing, as more than traces of lipase in serum have never been demonstrated".

Another suggestion is that hyperlipaemia results from anoxaemia. Following their original observations in the exsanguinated rabbit, Boggs and Morris (1909) stated that "lowered oxidation following great loss of red cells may play a part". Milne (1912-13) favoured the same explanation, and Starup (1934a) considered that all types of anaemic hyperlipaemia were referable to oxygen deficiency as the ultimate cause. He was able to induce a marked hyperlipaemia in rabbits by maintaining them in a reduced atmospheric pressure, and Schwartz and Lichtenberg (1937), although unable to explain the mechanism responsible, obtained evidence to suggest that hyperlipaemia in bled rabbits is caused by the loss of red cells only. On the other hand, MacLachlan (1939) was unable to demonstrate the condition in dogs or cats kept at a reduced oxygen tension for six hours, and Muller and Talbott (1931) could find no evidence of increased blood fat in man at high altitudes; but once again species differences must be remembered.

It therefore seems that while anaemia frequently brings about an increase in plasma neutral fat, the means by which it does so has not yet been satisfactorily explained. Anaemic anoxia must be considered a likely cause of the hyperlipaemia in the weanling mice.

Thyroid gland. It has been repeatedly demonstrated that the activity of the thyroid gland affects serum lipids. In particular, the level of plasma cholesterol is constantly increased in hypothyroidism of whatever origin (Remond, Colombies and Bernardbeig, 1924; Baumann and Holly, 1925-6; Werner, 1929; Mason, Hunt and Hurxthal, 1930; Westra and Kunde, 1931-2, 1933; Hurxthal, 1933a; Bronstein, 1933; Gilligan, Volk, Davis and Blumgart, 1934; Parhon and Ornstein, 1935; Chamberlain, Jacobs and Butler, 1936; Turner, Present and Bidwell, 1938; Fleischmann, Schumacker and Wilkins, 1940; Thompson and Long, 1941; Hurxthal and Simpson, 1941; Peters and Man, 1943,[b]

1950; Handler, 1948; Foote and Merivale, 1949). In hyperthyroidism, on the other hand, many of the above reports indicate that cholesterol and other lipid components of the serum tend to fall, although this appears to be a less regular sequel than is the hypercholesterolaemia of hypothyroidism.

A number of these authors, and also others such as Heckscher (1925a, b), Nicholls and Perlzweig (1928), Boyd and Connell (1936), Gildea, Man and Peters (1939) and Man, Gildea and Peters (1940) have reported that, in thyroid disease, some other lipid substances in the serum roughly parallel the concentration of cholesterol. However, Man et al. (1940) and Peters and Man (1943, 1950b) do not agree that the serum neutral fat concentration is influenced by thyroid activity, although Chaikoff, Entenman, Rinehart and Reichert (1941) found that it was definitely high in thyroidectomised dogs. This rise was dependent, however, on a sufficient caloric intake (Entenman, Chaikoff and Reichert, 1942a). Recently Clément (1950) has stated his opinion that there are such conflicting views on the effect of the thyroid gland on fat mobilisation that the question must be left open.

That the hyperlipaemia seen in clinical hypothyroidism is a specific effect of deficiency of the thyroid hormone is clear from various reports that the abnormality disappears with replacement therapy (Hurxthal, 1933b, 1934; Gilligan et al., 1934; Gildea et al., 1939). The same result can be achieved in experimental cretinism (Westra and Kunde, 1931-2, 1933). Epstein and Lande (1922) noted that the level of serum cholesterol varied inversely with the rate of basal metabolism and suggested that the relationship was more than incidental. This has been disputed by Gardner and Gainsborough (1928), and Blumgart and Davis (1934) showed that following thyroidectomy the B.M.R. reached its lowest level within two months, whereas the serum cholesterol concentration continued to increase for some months longer. Cutting, Rytand and Tainter (1934), and, recently, Stamler, Silber, Miller, Akman, Bolene

and Katz (1950) have shown that while the administration of dinitrophenol increases the B.M.R., yet, unlike desiccated thyroid, it does not affect plasma lipid levels; and Stamler et al. therefore concluded that the effects of the thyroid hormone on plasma lipids "are not mere non-specific by-products of the induced generalised increase in metabolic rate". Others have come to the same conclusion (Gildea et al., 1939; Man et al., 1940).

Although the connection between thyroid activity and plasma lipid response is not understood, there can be no question of its existence. How far neutral fat is involved is doubtful, but it can be concluded that the influence of the thyroid gland must be considered in studying the hyperlipaemia observed in the weanling mice.

Pituitary gland. There can be no doubt that the anterior lobe of the pituitary gland affects fat metabolism directly or indirectly, although claims that it secretes a specific "fat metabolism hormone" (Anselmino and Hoffman, 1931; Anselmino, Hoffman and Rhoden, 1936) have not been substantiated. Foglia and Mazzocco (1938) have reported that hyperlipaemia can follow upon the administration of anterior pituitary extracts, and liver lipids may be increased, and ketonaemia induced, by similar means (Burn and Ling, 1930; Black, Collip and Thomson, 1934; Butts, Cutler and Deuel, 1934; Best and Campbell, 1936; MacKay and Barnes, 1937a, b; Neufeld and Collip, 1939). There is evidence, however, that these effects are mediated through the diabetogenic hormone, and are only special features of the effects of carbohydrate starvation (Peters and van Slyke, 1946).

Hypophysectomy alone appears to have but little effect on the blood lipids of the dog (Chaikoff, Gibbs, Holtom and Reichert, 1936). However, the same school of workers (Entenman, Chaikoff and Reichert, 1942b) have shown that if hypophysectomy is superimposed upon thyroidectomy the blood lipids rise to very

high levels, provided that caloric intake is maintained. This result they ascribe to depression of whatever accessory thyroid tissue may have remained after the operation.

There is therefore no good evidence that the pituitary gland has a direct effect on fat metabolism; probably its influence is restricted to indirect effects on the thyroid gland and the pancreatic islet cells. Indeed, Chamberlain, Jacobs and Butler (1936) go so far as to state that there is no evidence that the level of total blood lipids is materially affected by any endocrine disorder except diabetes mellitus, provided the thyroid gland is not demonstrably involved. There is also a total lack of evidence that dietary calcium carbonate effects the pituitary gland, and it can therefore be concluded that it is most unlikely that the hyperlipaemia in the weanling mice from the high calcium carbonate groups could be related to the influence of the hypophysis.

Gonads. Although Randall (1940) has claimed that repeated injections of testosterone over a period of weeks will raise the level of serum lipids in the human subject, several other workers have reported that the testis and its hormone are without influence on blood lipids (Kochakian, MacLachlan and McEwen, 1938; Looney and Romanoff, 1940; Entenman, Lorenz and Chaikoff, 1940; Groetsema and Marlow, 1941).

On the other hand, it is well-established that oestrogenic substances can induce hyperlipaemia in rats, guinea-pigs and chicks (Lorenz, Chaikoff and Entenman, 1938a, b; Lorenz, Entenman and Chaikoff, 1937-38; Entenman, Lorenz and Chaikoff, 1938; Bogdanovitch and Man, 1938; Zondek and Marx, 1939; Loeb, 1942; Riddle, 1942; Gardner and Pfeiffer, 1943; Horlick and Katz, 1948). A number of these reports also indicate that body fat storage is increased. Thyroid administration seems to oppose this effect (Fleischmann and Fried,

1944, 1945; Stamler, Miller, Akman, Silber, Bolene and Katz, 1950); and Stamler et al. go on to point out that "the interrelationships between thyroid and estrogenic hormones extend beyond lipid metabolism, and apparently operate in many physiologic situations ....."

However, it is difficult to perceive a connection between the hyperlipaemic mice and hyperoestrogenism. Milky serum was observed in males and females alike, so that, if any such endocrine disorder were involved, presumably it must have originated in the dams and probably have been referable to the thyroid gland.

Pancreas. The association of hyperlipaemia with diabetes mellitus - caused by a deficiency of insulin, the hormone of the pancreas - has long been recognised (Fischer, 1903; Bloor, Gillette and James, 1927; Man and Peters, 1934, 1935; Hirsch and Carbonaro, 1950). The neutral fraction appears to show the greatest elevation (Herbert, 1935). Undoubtedly the hyperlipaemia results from the mobilisation from the depots of large amounts of fat, which are used to replace carbohydrate for energy production. As carbohydrate combustion is minimal in diabetes, large amounts of ketones are formed in the liver, and under these circumstances hyperlipaemia and ketonaemia can both reach extremely high levels, particularly after acidosis has ensued.

Although there had been nothing other than hyperlipaemia to suggest the existence of diabetes mellitus in the experimental mice, yet at the same time there was no positive evidence to exclude such a possibility. I therefore concluded that the point deserved further investigation.

SUMMARY AND CONCLUSIONS

The various factors known to be capable of causing fatty livers have been reviewed. These include starvation; diabetes mellitus; deficiencies of

unsaturated long-chain fatty acids, inorganic phosphates, choline, methionine and inositol; excess of biotin, thiamin, riboflavin and nicotinic acid; liver damage, including damage by protoplasmic poisons; 'pathological adiposity'; anoxia; and endocrine disorders. Consideration of these factors has suggested that the most likely causes of the liver damage seen in the experimental mice were diabetes mellitus, anaemic anoxia and hypothyroidism; but a superimposed deficiency of a lipotropic agent such as choline or inositol could not be ruled out, and in some cases semi-starvation may have played a part.

The known causes of hyperlipaemia have also been reviewed. Apart from certain diseases peculiar to the human subject, and others which obviously did not apply to the experimental mice in question, these causes include the ingestion of food rich in fat; pregnancy and lactation; ether anaesthesia; avitaminosis B; starvation and malnutrition; various types of anaemia; and certain endocrine disorders. The factors most likely to have been concerned with the hyperlipaemia affecting the experimental mice were considered to be anaemia, hypothyroidism and diabetes mellitus. Ether anaesthesia or starvation may have increased the severity of the disorder, but could not have been responsible for the differences between diet-groups.

(iii) Reproduction

The inconclusive nature of the breeding trial is not altogether surprising. The variability of reproduction data is normally so great in small laboratory animals that all but the strongest treatment effects must remain undetected without the use of large numbers of experimental animals. To say this does not admit that any effects which the supplements may have had on reproduction must necessarily have been unimportant; and, indeed, differences were often quite large in this experiment. For example, with first litters the higher level of

calcium carbonate was associated with reductions of 18% in the number born, 20% in the number weaned, and 17% in the total weight weaned; if these figures could be regarded as reliable, then the effects of the supplement would have been quite considerable. The difficulty, however, is that in using criteria such as these, variability due to random causes may lead to equally large differences, and, unless it is reduced to smaller proportions by the use of larger numbers of animals, there can be no reasonable certainty that any differences were not due to chance.

Nevertheless, the trends noted in the effects of calcium carbonate were in complete agreement with Richards' results in Experiment 1, and also with those of Howie and Porter (1950); in both these instances, large numbers of animals were used for direct comparison. It is notable that, in both these experiments as well as in the present one, the effects of the supplement were more apparent on rearing performance than on fertility or fecundity. It is also worth remembering that birth is only an incident - albeit an important one - between conception and adult life, and the effects on fertility and fecundity could have been due to the same inherent cause as the effects on rearing; in that case they would have been accounted for by embryo-death, and it is a pity that no simple means exists for estimating the importance of this factor.

Properly controlled laboratory experiments on the relation of the calcium intake and the Ca : P ratio of the diet to reproduction are very few in number, but the fundamental work of Sherman and his colleagues must be mentioned. Sherman and Muhlfeld (1922a, b) and Sherman and Campbell (1924, 1929-30) compared the Sherman A and B diets from many points of view, including various aspects of reproduction, and found the B diet invariably superior over several generations. They believed that this was because the B diet (which contained about 66% of dried milk) had a higher calcium content than the A diet (with only about 16% of dried milk); and Sherman and Campbell (1935) obtained an improvement in the

performance of rats on the A diet when they raised its calcium content from 0.2% to 0.35% with a supplement of calcium carbonate. In an extensive experiment carried out with rats, Cox and Imboden (1936) used inorganic salts to vary the Ca : P ratio of a synthetic diet from as little as 0.1 : 1 up to as much as 10 : 1. They found that the optimum ratio for gestation and lactation was dependent on the absolute amounts of calcium in the diet, and increased with increasing calcium intake. Although neither the proportion of fertile to total matings nor survival rate during rearing were studied by the authors, they can be worked out from the data presented and lead to similar conclusions with regard to the Ca : P ratio of the diet. The optimum conditions for reproduction, in the opinion of these workers, are a Ca : P ratio of unity with a calcium intake of 0.49%. This conclusion agrees well with the findings reported in the present experiment.

These results are particularly interesting in view of the theory recently advanced by Hignett (1949, 1950) that excessive dietary calcium may under certain circumstances play a part in causing infertility in cattle. They should not, however, be accepted as providing very strong supporting evidence for the theory, at least until more is learnt of the *modus operandi* involved. There is a vast difference in physiology between the reproductive systems of mice and of cattle (Asdell, 1946). In any case, Hignett's proposition is confined to the question of fertility, and in these experiments fertility was one of the least affected aspects of reproductive performance. Further, Hignett contends that the CaO : P_2O_5 ratio of the diet is of great importance, and that, provided there is an adequate intake of phosphorus, it should not be much wider than 1:1 (*i.e.* Ca : P = about 1.6 : 1). He also holds that an induced deficiency of phosphorus is probably the ultimate cause of the disorder. In the experimental mice, however, the phosphate intake was already high, and restoration of the Ca : P ratio (from 2.3 : 1 to 1.1 : 1) with additional phosphate did not neutralise the harmful

effects of the additional calcium carbonate. It therefore seems probable that - assuming Hignett's views to be well-founded - two separate mechanisms are involved.

On the other hand, increasing the calcium content of the ration has been found useful in overcoming reproductive disorders in some domestic animals, particularly pigs. Baskett (1926) observed an increase in the number of pigs born as well as the number weaned when a calcium supplement was fed to breeding sows. Recently, Pullar (1950) reported an improvement in the reproductive performance of sows when a mineral supplement containing calcium was fed. The chief abnormalities which have been reported as responding to an increased calcium intake are abortion, stillbirths, extreme difficulty in farrowing, and failure to commence or to maintain lactation (Evans, 1929; Davidson, 1930; Hogan, 1932). In most of these cases, however, the diet has been frankly deficient in calcium and has had a very low Ca : P ratio. The paucity of reliable information on the relation between the calcium content of the diet and reproductive processes is illustrated by the fact that this subject receives very little attention in reviews on nutrition and reproduction (Friedman and Turner, 1939; Sutton, 1941; Huffman and Duncan, 1944; Asdell, 1949; Reid, 1949; Durrell, 1951).

The results of the experiment also suggest that the iron supplement may have brought about an improvement in reproductive performance, particularly with second litters. While there is no certain indication of the means by which the improvement may have been effected, yet it is a remarkable fact that ferric citrate and calcium carbonate opposed one another in their effects on reproduction as well as in many other ways. It is therefore very tempting to suggest that the impairment in reproduction associated with the higher level of calcium carbonate was due, as was the impairment in blood values, to an induced deficiency of iron, and that the additional iron rectified this deficiency. It

is true that iron-deficiency anaemia is not generally recognised as a common cause of infertility, although Sundelin (1940) has claimed that anaemia interferes with ovarian function in the rat. Nevertheless, the infants of iron-deficient mothers are usually iron-deficient themselves (Scott, 1923; Mackay, 1931, 1935; Alt, 1937, 1938; Strauss, 1933; Parsons and Hawksley, 1933), and it seems by no means unreasonable to suggest that this would result in a higher infant mortality rate and a lower body weight at weaning. Indeed Mackay (1935) states that iron-deficiency anaemia may double the morbidity-rate of human babies, and Parsons, Hickmans and Finch (1937) reported not only that the reproductive powers of iron-deficient rats were impaired but that the young were reared only with difficulty and were subnormal in weight and size. But although there is a lot to be said for this suggestion, and very little against it, it cannot be considered proved from this experiment alone.

(iv) Thymus gland

The function of the thymus gland - if indeed it has one at all - is not understood. It is therefore not easy to appreciate the true significance of the atrophic changes observed in the thymus glands of the weanling mice from the high calcium carbonate groups.

In the normal animal the thymus increases in size up to approximately the time of sexual maturity. Numerous attempts have been made to influence tissue development and sexual function by means of extracts prepared from the organ (see Park and McLure (1919) for a comprehensive review of the earlier work), but little definite information of a positive nature has emerged. Riddle and Křůženecký (1931) were unable to detect any change in the development or function of the reproductive organs following thymectomy, but Rowntree, Clark and Hanson (1934) claimed that a thymus extract accelerated the growth and development of

rats, and hastened the onset of adolescence in their offspring; and Rowntree, Clark and Steinberg (1934) (who also reviewed earlier work in this field) stated that thymectomy of parent rats slowed the rate of growth in the young. However, Hammond and Bird (1942) pointed out that the findings of Rowntree et al. (1934, 1935) could be explained on the grounds of selective breeding. These workers found experimentally that the thymus glands of rapidly-growing chicks were several times as large in proportion to body weight as those of slowly-growing chicks, and also observed that growth rate was not affected by thymectomy. They thus concluded that the large thymus glands were the result and not the cause of the fast rate of growth.

It is now generally believed that the thymus is not an endocrine gland (though Comsa, 1949a, b has recently claimed that it has an anti-thyroid function), and certainly it is agreed that its presence is not essential to life. Majority opinion now holds that the thymus does not affect the sex organs, but at the same time there is no doubt that the reverse is not true. In both males and females the organ is sensitive to castration or to the injection of sex hormones, which respectively increase and decrease it in size at all ages (see Ross and Korenchevsky (1941) and Flagge (1941) for bibliography). In fact, the secretion of gonadal hormones at sexual maturity almost certainly accounts for the fact that at about this time the thymus normally ceases to grow and starts to become involuted.

Embryologically, the thymus is an epithelial organ, being later invaded by mesodermal cells which form thymocytes. There is some doubt whether these cells differ from ordinary lymphocytes, but modern opinion appears to be that they do not (see Downey (1948) for discussion). The epithelial cells persist in the form of reticular cells and, in some species, as Hassall's corpuscles, but the latter are very few and small in the rat (Hewer, 1915-16; Ross and Korenchevsky, 1941) and in the mouse (Greig, unpublished observations). Histologically, the

thymus of a normal immature animal consists of two distinct portions, a dense outer cortex and a less dense medulla, the thymoctyes being bound together by the stellate, anastomosing reticular cells. The organ also contains occasional cells of plasma cell and mast cell types.

When involution commences at or just after sexual maturity (so-called 'physiological' or 'age' involution), the thymocytes begin to disappear and there is a gradual replacement of the thymus tissue by adipose and fibrous tissue. In old age, atrophy of the thymus has become extreme, although, as Crotti (1938) has pointed out, the presence of mitotic figures in the senile thymus indicates that the organ is still functioning. In a survey of 496 rats, Plagge (1941) found the first signs of atrophy at 60 days of age, the average age of sexual maturity having been reached at 55 days for males and 45 days for females. As involution proceeded, the two lobes of the gland began to separate and to become surrounded by adipose tissue; they became diminished in thickness but not decreased in length. Histological signs of involution were not seen until about 200 days of age when the thymocytes in the cortex began to become thinned, and the boundary line between cortex and medulla began to fade. In extreme involution, the medulla extended almost to the periphery of the gland, and also became thinned in thymocyte concentration and sometimes partly replaced by fat.

A second type of involution ('accidental' involution) may be seen in some young animals. Accidental involution is generally considered to be a sign of non-specific stress, and is seen in shock, inanition, cachectic or infectious diseases, and in malnutrition. Though some authors (e.g. Marine, 1932) claim that there are no definite distinguishing features between the two types, others maintain that accidental involution is characterised by a decrease, rather than an increase, in the fatty tissue associated with the organ, and also by the fact that the boundary line between cortex and medulla becomes less clearly defined, or even lost, before involution has become extreme (Hammar, 1927; Smith and

Ireland, 1941).

The thymus atrophy in the weanling mice from high calcium carbonate groups, together with the histological changes seen in these organs, clearly indicate that accidental involution had occurred. Since this condition is usually regarded merely as a symptom of non-specific stress, it seems probable that the anaemia, the slow rate of growth, and the generally poor development of these animals were responsible for the abnormalities. At the same time, it is worth noting that Richards (1949a) observed thymus atrophy in pyridoxin-deficient rats, especially those receiving a supplement of calcium carbonate, and that the deficit in thymus weight was much greater than might have been expected from the deficit in body weight (see Stoerk, 1946). While it is rather doubtful whether this could be held to imply that pyridoxin deficiency has a specific effect on the thymus gland, it is interesting to remember not only that the effects of pyridoxin deficiency are accentuated by the addition to the diet of calcium carbonate, but also that such a deficiency can result in anaemia (see p.177). There are therefore some *prima facie* grounds for suggesting that the abnormal young mice may have been deficient in pyridoxin.

(v) Heart

The oedema in the heart muscle calls for little comment, as oedema was generalised throughout the body in the anaemic animals. It is perhaps more remarkable that no evidence of fatty change was observed in the heart, as this is a frequent lesion in severe anaemia in some other species, notably man; indeed, the liver appears to have been the only organ so affected.

The increased size of the heart in the anaemic animals also calls for little comment; undoubtedly the enlargement was a compensatory hypertrophy associated with the lowered oxygen-carrying capacity of the blood. But it is interesting to

observe how closely this hypertrophy paralleled the degree of anaemia, especially in the growing animal. No previous report of such a correlation has been found in the literature. Evidently the body seeks to maintain a particular rate of oxygen flow, and once this has been achieved the heart ceases to hypertrophy further; that is to say, the stimulus to cardiac hypertrophy is not directly dependent upon the fact of the existence of anaemia, but is governed by the difference between the body's oxygen requirements and the supplies reaching the tissues.

C. PROPOSALS FOR FURTHER WORK

The results of Experiments 1 and 2 have shown that the major effects of supplementing the diet of breeding mice with calcium carbonate are as follows:

(1) the development, in both mothers and young, of hypochromic microcytic anaemia accompanied by compensatory hypertrophy of the heart;

(2) impairment in reproduction performance, especially as regards rearing of the young;

(3) the appearance of hyperlipaemia in the young mice, accompanied by fatty change in the liver;

(4) accidental involution of the thymus gland in the young animals.

It has also been shown that the position may be largely restored by the further addition to the diet of a supplement of ferric citrate; but, although all of the above abnormalities could be explained by a deficiency of iron, it has nevertheless not been conclusively proved that the calcium carbonate had in fact induced such a deficiency.

The four groups of major effects listed above will now be considered individually.

(1) Anaemia. If the anaemia was not caused by iron deficiency, it must have been due to some other factor capable of inducing microcytic hypochromic anaemia in association with a hyperplastic bone marrow. The factors known to possess such properties, and which could have operated in this instance, have already been mentioned (p. 42). They include (1) deficiencies of copper and possibly cobalt (claims have also been made for deficiencies of other metals), and (2) deficiency of pyridoxin. There also remains the question of hormonal disorders.

With the possible exception of hyperthyroidism, however, none of these appears to result in a hyperplastic bone marrow; and only hypothyroidism (including hypopituitarism affecting the thyroid gland through deficiency of the thyrotrophic hormone) has been associated with microcytic hypochromic anaemia - and even then only occasionally.

I therefore decided to explore each of these possibilities in turn.

2. Reproduction. It has been shown that the impairment in rearing performance could very well be wholly accounted for by iron deficiency. It is true, however, that at various times deficiencies of other substances have been held responsible for impaired lactation and inability to rear young. The most important of these is manganese deficiency; deficiencies of pyridoxin and inositol have also been held responsible for such consequences, but indeed it is probable that many other dietary disorders or imbalances leading to loss of weight and cachexia could also lead to a poor rearing performance.

There is some evidence, however, that the availability of each of the three substances mentioned could have been interfered with by dietary calcium carbonate, and accordingly I decided to examine each of them more fully.

3. Fatty livers and hyperlipaemia. The possible causes of these abnormalities have been discussed in some detail, and it has been concluded that possible causal factors which could not be excluded from consideration include anaemia, hypothyroidism, deficiency of lipotropic factors (choline or inositol), and diabetes mellitus.

I decided, therefore, to examine each of them in turn.

4. Thymus gland. The thymus atrophy has been shown to be the result of

accidental involution. While this was probably merely a symptom of stress, possibly associated with the anaemia and the consequent poor bodily development, evidence has also been led to suggest that pyridoxin deficiency may have been responsible.

I therefore decided to examine the thymus glands of young mice born of mothers given a supplement of pyridoxin in addition to being fed a diet high in calcium carbonate.

My studies of all these factors, and the experimental work connected with them, are described in the remaining sections of Part II of this thesis.

iron salts prepared from 'pure' reagents were not (Waddell, Elvehjem, Steenbock and Hart, 1928b). Hart, Steenbock, Waddell and Elvehjem (1928) then found that a liver preparation cured the anaemia, and fractionated it. The active material was found in the hydrogen sulphide precipitate, and was therefore suspected to be copper; and when copper sulphate was fed together with pure ferric chloride it proved effective against the anaemia. Next, Waddell, Steenbock and Hart (1929a) reported that the so-called 'pure' iron preparations were of some value in combating the anaemia if fed at very high levels, but that this value was lost if the salts were freed from copper contamination.

Waddell, Steenbock and Hart (1929b) then tested the effects of twelve other elements as adjuncts to iron; these were zinc, chromium, germanium nickel, cobalt, lead, antimony, tin, cadmium, mercury, arsenic and manganese. None approached copper in effectiveness, so that it was concluded that copper was unique in possessing this haemopoietic activity.

In the meantime, however, these claims had been hotly challenged. Myers, Beard and their colleagues reported that a whole series of elements (such as copper, nickel, germanium, manganese, titanium, zinc, rubidium, vanadium, chromium, selenium, mercury and arsenic, but not cobalt, magnesium or aluminium) assisted iron in promoting haemoglobin generation, and that several of these were equally as effective as copper for this purpose (Beard and Myers, 1928-9, 1930, 1931-2; Myers and Beard, 1929, 1931-2; Beard, Rafferty and Myers, 1931-2; Myers, Beard and Barnes, 1931-2). They thus queried the specificity of copper, and indeed found that in some cases inorganic iron alone sufficed (Eveleth, Bing and Myers, 1932-3). Mitchell and Miller (1929) reported that inorganic copper was less successful than spinach ash, though later they agreed (Mitchell and Miller, 1931) that copper was essential to haemoglobin formation. Others who queried the specificity of copper in erythropoiesis were Titus and Cave (1928), Titus, Cave and Hughes (1928), Titus and Hughes (1929) and Drabkin and Waggoner (1930).

On the other hand, the views of the Wisconsin group were supported by a great weight of experimental work, such as that of Keil and Nelson (1930-1, 1931, 1932), McHargue, Healy and Hill (1928), Cunningham (1931), Underhill, Orten and Lewis (1931), Orten, Underhill and Lewis (1932), Lewis, Weichselbaum and McGhee (1929-30), Becker and McCollum (1930), Geraghty, Underhill, Orten and Lewis (1932-3), Fitzhugh, Robson and Drabkin (1933), and Stein, Radetsky and Lewis (1936).

The evidence available now is so strong that there can be no doubt that copper plays a specific part in erythropoiesis (Elvehjem, 1932b; Hutchison, 1938; Schultze, 1940). This fact has been shown in many species besides rats and rabbits, including mice and pigs (Elvehjem and Hart, 1932; Schultze, Elvehjem and Hart, 1936a; Gubler, Lahey, Chase, Cartwright and Wintrobe, 1952), dogs (Elden, Sperry, Robscheit-Robbins and Whipple, 1928; Potter, Elvehjem and Hart, 1938; Frost and Elvehjem, 1939; Frost, Potter, Elvehjem and Hart, 1940; Maass, Michaud, Spector, Elvehjem and Hart, 1944), cattle (Neal, Becker and Shealy, 1931) and poultry (Elvehjem and Hart, 1929). Considerable controversy has existed in regard to the need for copper in erythropoiesis in the human subject. Most of the evidence is clinical rather than experimental, so that while some authors (Heath, 1933; Mackay, 1933; Davidson, 1933; Wilkinson, 1933; Lottrup, 1934; Bethell, Goldhamer,

Isaacs and Sturgis, 1934; Barer and Fowler, 1937) have found copper and iron together to be not superior to iron alone in the treatment of hypochromic anaemias, yet many others (Mills, 1930, 1931; Adamson and Smith, 1931; Josephs, 1931; Lewis, 1931; Parsons and Hawksley, 1933; Dameshek, 1933; Maurer, Greengard, Curtis and Klüver, 1934; Elvehjem, Siemers and Mendenhall, 1935; Goldstein, 1935; Usher, MacDermot and Lozinski, 1935; Elvehjem, Duckles and Mendenhall, 1937; Hutchison, 1938; Lucas and Summerfeldt, 1939) have found it of benefit in some cases (especially in children), and this fact indicates that probably the human being, like other species, utilises copper for haemoglobin formation.

Copper is not itself a constituent of haemoglobin, although it can be isolated from erythrocytes (Elvehjem, Steenbock and Hart, 1929a; Tompsett, 1934; Keilin and Mann, 1944). It appears to exercise its erythropoietic function chiefly by catalysing the synthesis of the haemoglobin molecule (Keil and Nelson, 1930-1; Josephs, 1931; Elvehjem and Sherman, 1932; Elvehjem, 1932a, b, 1935; Parsons, 1933; Hutchison, 1938). Its precise action in this respect is not clear. Cunningham (1931) suggested that it may catalyse the formation of a ferro-porphyrin, but emphasised that it plays no part in porphyrin synthesis itself - a point confirmed by the work of Schultze (1942). Elvehjem (1932a) stated that copper "acts in the conversion of iron into forms which can be used for the construction of the haemoglobin molecule", while Muntwyler and Hanzal (1933) and Hutchison (1938) believed that it may assist in the mobilisation of iron from the storage depots.

There is much evidence to show that copper plays no part in the absorption, storage or excretion of iron (Cunningham, 1931; Josephs, 1932, 1939b; Elvehjem and Sherman, 1932; Elvehjem, 1932a, 1935; Scott and McCoy, 1944); but very recently Chase, Gubler, Cartwright and Wintrobe (1952) and Gubler, Lahey, Chase, Cartwright and Wintrobe (1952), working with rats and pigs respectively, reported that the amount of iron absorbed was influenced by the amount of copper in the tissues. The latter group of workers, however, agreed that copper also has a more direct effect on erythropoiesis.

Additional effects of copper on erythropoiesis have also been suggested by other investigators. For instance, Kletzien, Buchwald and Hudson (1932-3) considered that, in the rat, the presence of copper increases the utilisation of iron by tissues other than blood, though Josephs (1932) obtained the opposite result in human infants. Others (Stein and Lewis, 1931-2, 1933; Smith and Medlicott, 1944) considered that, besides a catalytic effect on haemoglobin formation, copper also has a stimulating effect on the release of reticulocytes from the marrow; but Schultze and Elvehjem (1933) and Schultze (1940) have pointed out that although the administration of copper gives rise to a reticulocytosis, the production of reticulocytes may depend on the presence of newly-formed haemoglobin or its precursors, so that the reticulocytosis observed with copper may be only a secondary effect. Robscheit-Robbins and Whipple (1942) found that the addition of copper to the diet of exsanguinated dogs effected a moderate, though irregular, increase in haemoglobin production, even although the animals had adequate stores of copper in their livers and spleens; from this they adduced that copper may have had some effect on enzyme complexes related to globin formation. Another action of copper was suggested by Wickwire, Burge and Krouse (1936), who noted that copper deficiency resulted in an increased rate of red cell disintegration.

Copper is a universal constituent of protoplasm, and is found in greatest concentration in the foetus and young growing animal (Sheldon and Ramage, 1931; McHargue, 1925; Lindow, Petersen and Steenbock, 1929; Morrison and Nash, 1930), especially in the liver, kidney and bone marrow (Tompsett, 1935; Schultze and Simmons, 1942). It is known to be essential, and specific, for the formation and maintenance of a number of porphyrin compounds besides haemoglobin, notably respiratory pigments with a haem nucleus such as cytochromes a, b and c (Cohen and Elvehjem, 1934; Schultze, 1939, 1941, 1942) and catalase (Schultze and Kuiken, 1941). It is also known to be a constituent of some enzymes, such as haemocuprein (Keilin and Mann, 1944) and ascorbic acid oxidase (Ramasarma, Datta and Doctor, 1940; Hochberg, Melnick and Oser, 1950).

The anaemia of copper deficiency, like that of iron deficiency, is microcytic and hypochromic (Fitzhugh, Robson and Drabkin, 1933; Smith, Medlicott and Ellis, 1944) and is associated with hypocupraemia (Schultze, Elvehjem and Hart, 1936b). Anaemic infants also have a lowered level of liver copper (Morrison and Nash, 1930; Chou and Adolph, 1935). Gubler, Lahey, Chase, Cartwright and Wintrobe (1952) have shown that the anaemias of copper deficiency and iron deficiency, besides being similar in respect of the morphological characters of the red cells, have many other features in common, such as a normoblastic hyperplasia of the bone marrow, hypoferraemia, and an increased iron-binding capacity in the plasma.

To summarise, it may be concluded that copper, besides being necessary for the enzymatic functions of intra-cellular respiration, plays an essential part in the process of erythropoiesis by catalysing some stage of haemoglobin synthesis, and that it cannot be replaced for this purpose by any other element. Copper may also play some additional part in blood formation, particularly in the release of reticulocytes from the bone marrow, and it may also influence iron absorption to some extent. A deficiency of copper results in an anaemia indistinguishable morphologically and in other ways from the anaemia of iron deficiency.

There is thus good *prima facie* evidence that the anaemia seen in Experiment 2 could have been due to a deficiency of copper and not of iron. But, as will now be seen, further examination of the question diminishes the likelihood that this had been the case.

The requirement of copper for erythropoiesis is very low (as shown by the work, described above, of Hart and his collaborators). The dietary

requirement of mice does not seem to be known exactly, and no doubt depends on, among other things, the availability of the copper in the food, but the absolute intake of the experimental mice was probably adequate. Although cows' milk is low in copper (McHargue, 1925; Lindow, Elvehjem and Petersen, 1929; Elvehjem, Steenbock and Hart, 1929b), storing or heating milk in the presence of metallic copper, or even passing it through copper pipes, increases its copper content (Supplee and Bellis, 1922), and it has been shown that copper is taken up in considerable quantities in the manufacture of condensed or evaporated milk (Rice and Miscall, 1923; Quam and Hellwig, 1928; Stein and Lewis, 1931-2, 1933). Since the diets fed to the experimental mice contained nearly 33% of evaporated milk, it would seem that the absolute intake of copper in the milk must have been more than negligible.

Copper is also a constituent of nearly all foods (Lindow et al., 1929) including wheat, which formed nearly 66% of the mouse diets. The average copper content of wheat is given by McCance, Widdowson, Moran, Pringle and Macrae (1945) as 0.60 - 0.65 mg.%, while Lindow et al. (1929) state that wheat bran contained 12.1, and wheat germ 14.2 mg. of copper per Kg. of dry matter. From this it can be concluded that the total copper intake of the mice should have been quite considerable. Spectrographic analysis* of diet 81 confirmed this expectation; there were about 0.275 mg. of copper (as Cu) in each 100 g. of undried diet.

Spectrographic analysis* of the diet after supplementation with the iron citrate preparation showed, however, that its copper content was now increased by fully 50%. The iron citrate must, therefore, have been quite heavily contaminated with copper. Consequently, although the possibility appeared rather remote, it nevertheless seemed wise in view of this last finding to determine whether the contaminating copper had been responsible for the action of

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* Kindly performed for me by Dr. R. L. Mitchell, of the Macaulay Institute for Soil Research, Aberdeen.

the iron supplement. This could be done either by feeding a copper supplement alone, or by feeding a completely copper-free iron preparation. Since the work of Hart and his colleagues had shown how very difficult it is to obtain an iron preparation completely devoid of traces of copper, the first method appeared to be simpler, and certainly the more certain. Accordingly I decided to carry out such an experiment, which is described later as Experiment 3.

Manganese

There is good evidence that manganese is essential for health. It is found in blood, liver, pancreas, kidney and suprarenal glands, and to a less extent in many other tissues (Sheldon and Ramage, 1931). In laboratory animals the effects of manganese-deficient diets have been seen chiefly in disorders of reproduction and lactation.

Daniels and Hutton (1925) and Mitchell and Schmidt (1926) found that combinations of minerals including manganese improved the reproduction performance of milk-fed rats. Orent and McCollum (1931) fed breeding rats on a manganese-deficient diet; the male rats developed atrophy of the testis, but the females had normal oestrous cycles and readily conceived although they showed lack of readiness to suckle their young. On the other hand, Kemmerer, Elvehjem and Hart (1931) observed that female mice fed on whole cows' milk with supplements of iron and copper did not maintain a normal rhythm in their oestrous cycles, but that the addition of traces of manganese to the milk corrected this and also had a favourable effect on growth. Waddell, Steenbock and Hart (1931) obtained similar results with rats. These various reports conflict only in respect of the severity of the disorders encountered, and this could easily be explained on the basis of different degrees of deficiency.

In the preceding section it was stated that a number of authors have claimed that other elements besides copper assisted in the synthesis of haemoglobin; in most of these cases the listed elements included manganese, but Titus, Cave and Hughes (1928), Titus and Cave (1928) and Titus and Hughes (1929) attached especial importance to the value of this metal. However, all these claims for manganese were decisively rejected at the time by many other authors, including Waddell *et al.* (1929), Lewis *et al.* (1929-30), Keil and Nelson (1930-1, 1931, 1932), Krauss (1931), Kemmerer, Elvehjem and Hart (1931), Orent and McCollum (1931) and Mitchell and Miller (1931). No evidence has since been recorded to overthrow the conclusions reached by these latter groups of workers, and manganese has been found of no clinical value as an adjunct to iron in the treatment of hypochromic anaemia in the human subject (Wilkinson, 1933).

It thus appears that manganese deficiency may manifest itself in impaired reproduction, but that it has not been proved to be a cause of anaemia.

It is also interesting to note that a deficiency of manganese, resulting in the condition known as perosis, may be induced in poultry by a diet containing large amounts of calcium, especially when inorganic phosphate is also present in relative abundance (Wilgus, Norris and Heuser, 1937; Wilgus and Patton, 1939; Schaible and Bandemer, 1940, 1942).

In view of these facts, it seemed important to determine whether manganese deficiency had played any part in the impaired reproduction performance given by the mice receiving the high calcium carbonate supplement.

Cobalt

While cobalt undoubtedly can affect erythropoiesis, the manner of its action is not yet clear.

Attempts to produce in rats an experimental nutritional anaemia due solely to a deficiency of cobalt have proved unsuccessful (Lewis, Weichselbaum and McGhee, 1929-30; Underwood and Elvehjem, 1938), although Frost, Elvehjem and Hart (1941) may have done so in the dog. Beard and Myers (1928-9) and Myers, Beard and Barnes (1931-2) reported that cobalt could replace copper in catalysing the incorporation of iron into the haemoglobin molecule but their claims have not been substantiated (Waddell, Steenbock and Hart, 1929b; Underhill, Orten and Lewis, 1931; ~~1932~~; Berlin, Huff and Henessy, 1951).

That cobalt has some effects on erythropoiesis was first demonstrated unequivocally by Waltner and Waltner (1929), who administered cobalt to rats and observed a polycythaemia and a rise in blood haemoglobin concentration. This result has been confirmed many times in a variety of species, including the rat (Orten, Underhill, Mugrage and Lewis, 1931-2, 1932; Brand and Stucky, 1933; Berwald, Arseneau and Dooley, 1934; Marshall, 1935; Josland, 1936-7; Kato, 1937), the dog (Davis, 1938; Frost and Elvehjem, 1939; Frost, Potter, Elvehjem and Hart, 1940; Frost, Elvehjem and Hart, 1941), the mouse and the guinea pig (Sutter, 1934), the rabbit (Kleinberg, 1934; Kato, 1937) and poultry (Davis, McCullough and Rigdon, 1945; Schultze, 1940). Reports on the use of cobalt with iron in clinical cases of hypochromic anaemia in man are conflicting (Waltner, 1930; Kato, 1937; Baxter, 1939; Cronin, 1939).

The polycythaemia is caused by a true increase in the total number of red cells in the circulation, and is associated with an increase in blood

volume and in haematocrit reading (Orten, Underhill, Mugrage and Lewis, 1932-3a; Davis, 1940a; Stanley, Hopps and Hellbaum, 1946) although the calculated corpuscular indices are probably not significantly different from those of normal bloods (Frost, Spitzer, Elvehjem and Hart, 1941). However, Stanley et al. (1946) found the mean cell volume increased. There is a true hyperplasia of the bone marrow (Mascherpa, 1930; Kato, 1937; Gordon, Kadow, Finkelstein and Charipper, 1946) and also erythropoietic activity in the spleen and liver (Dorrance, Thorn, Clinton, Edmonds and Farber, 1943), together with reticulocytosis (Orten, 1935-6; Davis, 1938; Gordon et al., 1946) and a normoblastic invasion of the peripheral blood (Kleinberg, 1934). Mitosis of the erythroblastic marrow elements is especially marked if haemocytoblast development is artificially inhibited (Kleinberg, Gordon and Charipper, 1939). The polycythaemia appears to be due solely to an increased production of red cells and not to their diminished destruction, as the serum bilirubin concentration is not lower than normal (Orten, 1935-6) and the average life-span of the reticulocytes remains unchanged (Berlin, Huff and Henessy, 1951). Rats rendered polycythaemic with cobalt have an increased capacity for work under conditions of anoxia (Dorrance et al., 1943).

Even rats fed on low-protein diets develop polycythaemia when cobalt is administered (Orten and Orten, 1945), but the condition does not supervene in animals deficient in iron or copper (Orten, Underhill, Mugrage and Lewis, 1931-2, 1932). It has also been reported (Berwald, Arseneau and Dooley, 1934) that cobalt does not induce polycythaemia in splenectomised rats, but Orten (1934-5) showed that the spleen played no part in the process.

In larger doses cobalt has a toxic action on erythropoiesis, and also causes losses in body weight (Stare and Elvehjem, 1932-3; Frost, Spitzer, Elvehjem and Hart, 1941; Robscheit-Robbins and Whipple, 1942; Davis, McCullough and Rigdon, 1945). The effects of cobalt have been partially or wholly inhibited by various means, including the administration of manganese (Orten, Underhill, Mugrage and Lewis, 1932-3b; Kleinberg, 1934), whole liver, liver extracts or choline (Davis, 1938, 1939; Marshall, 1935-6; Frost, Spitzer, Elvehjem and Hart, 1941) and ascorbic acid (Barron and Barron, 1936-7; Davis, 1940b).

Cobalt may also effect erythropoiesis in other ways. In the first place, it is a constituent of the molecule of vitamin B12, which may be identical with the haemopoietic principle of Castle (Hall and Campbell, 1948) or may correspond to the extrinsic factor (Berk, Castle, Welch, Heinle, Archer and Epstein, 1948). It is therefore essential for erythropoiesis, and indeed will cure pernicious anaemia in man (Smith, 1948; Rickes, Brink, Koniusky, Wood and Folkers, 1948). Again, anaemia often accompanies wasting diseases in sheep and cattle caused by a deficiency of cobalt (Filmer, 1933; Filmer and Underwood, 1934; Underwood and Filmer, 1935; Corner and Smith, 1938; Bowstead and Sackville, 1939; McDonald, 1942; Stewart et al., 1942; Geyer, Rupel and Hart, 1945; Killham, 1941; Neal and Ahmann, 1937). In the opinion of Stewart and Holman (1944), "cobalt deficiency in itself does not cause anaemia, nor does it materially interfere with haemoglobin formation", and it has been shown that in the ruminant the digestive mechanism imposes an additional

requirement for the element (Gall, Smith, Becker, Stark and Loosli, 1948a, b; Marston and Lee, 1949), this requirement being unrelated to vitamin B_{12} (Becker, Smith and Loosli, 1949).

The polycythaemic response to cobalt, however, clearly arises from some quite separate cause; but the nature of this is still not clear. Cobalt appears to accelerate the turn-over of iron in the bone marrow, with a consequent increase in haemoglobin production (Copp and Greenberg, 1946). Wintrobe, Grinstein, Dubash, Humphreys, Ashenbrucker and Worth (1947) have shown that it causes polycythaemia and an increase in haemoglobin values even in rats affected with the anaemia of inflammation, though the hypoferraemia and increases in red cell protoporphyrin associated with this condition are not corrected. They concluded that their observations were "consistent with the hypothesis that cobalt favorably influences the utilization of iron for the synthesis of haemoglobin". An effect of cobalt on iron metabolism is also suggested by the report of Kato and Iob (1940), who found that the spleen and bone marrow contained less iron in dogs and rabbits fed cobalt and iron than in control animals given iron alone.

Barron and Barron (1936-7), who observed that cobalt sulphate markedly diminished the respiration of immature red cells in vitro, suggested that such cells were thrown into the general circulation as mature non-respiring cells, and replaced in the bone marrow by new immature cells. However, Warren, Schubmehl and Wood (1944) were unable to confirm this observation; they concluded that the mechanism still remained unexplained, but showed that it was independent of the peripheral innervation to the marrow and that the blood vessels in this organ exhibited no morphological changes. On the basis of its inhibition by ascorbic acid, Davis (1940b) suggested that cobalt interfered with a respiratory function dependent upon this vitamin. Griffith, Pavcek and Mulford (1942) discovered that several sulphur-containing amino-acids, especially cysteine, were antidotal to the action of cobalt, presumably by forming relatively inactive complexes with it, and suggested that "cobalt poisoning may be due to the fixation and loss of sulfhydryl compounds in tissues with resulting interference with oxidative mechanisms", and that such interference with cellular oxidation may be the stimulus for the development of the polycythaemia. These findings have been confirmed by Orten and Bucciero (1948), who also favour the view that the polycythaemia results from simulated cellular anoxia.

Studies on cobalt metabolism, making use of radio-active isotopes, have been made by Copp and Greenberg (1941), Greenberg, Copp and Cuthbertson (1943) and Berlin (1950). They show that the body retains very little injected cobalt, thereby confirming the results obtained by Askew (1937); but Kent and McCance (1941) have shown that once cobalt has reached the tissues its excretion is negligible. The bodies of rats fed on normal diets contain only about 0.005 mg. of cobalt (Josland and McNaught, 1938), and polycythaemia results when the entire body of a rat contains as little as 0.04 - 0.05 mg. (Stare and Elvehjem, 1932-3). These latter workers also observed toxic symptoms in young rats fed more than 0.6 mg. of cobalt daily, but older rats were not seriously affected by 1.0 mg. daily. Frost, Elvehjem and Hart (1941) considered that "a level of 0.15 mg. of cobalt per day is about the detectable polycythaemia-producing level in rats", and observed a polycythaemic response in dogs

with a daily dose of 2 mg./kg. body weight. Clearly the normal daily requirement for cobalt must be very small; Underwood and Elvehjem (1938) calculated that it was less than 0.006 mg. daily for a rat weighing 100 g.

From the above review, it can be concluded that cobalt has probably three separate effects on erythropoiesis. One of these is seen only in ruminants, and a second is due to the activity of vitamin B_{12}, deficiency of which results in hyperchromic macrocytic anaemia. Obviously neither of these effects could have borne any relation to the anaemia seen in the mice in Experiment 2. Although it has never been proved that a genuine nutritional anaemia can result from cobalt deficiency, cobalt in small dosage certainly produces, under a wide variety of conditions, an increased blood haemoglobin concentration and a marked polycythaemia. This is clearly the result of stimulation of the erythropoietic centres, particularly the bone marrow; possibly this follows upon some interference with cellular respiration, but there is also evidence that cobalt, by influencing the utilisation of iron, assists in the synthesis of haemoglobin.

Since cobalt has the property of affecting erythropoiesis, it seemed quite possible that the response observed with the iron citrate preparations used in Experiment 2 could have been due - in part at least - to contamination of the iron citrate with cobalt. There was also to be considered a possibility that the calcium carbonate supplement had induced an actual deficiency of cobalt; cobalt is known to be less soluble in the presence of large amounts of calcium carbonate, and so less available to plants (Boddie, 1947; Mitchell, 1951), and a similar effect could conceivably result within the digestive tract of an animal.

Accordingly, I decided to determine whether a cobalt salt would produce a response in mice similar to that given by the iron citrate preparation.

EXPERIMENT 3

This experiment set out to determine whether or not any of the beneficial effects on erythropoiesis and reproduction, given in Experiment 2 by the addition of a preparation of ferric citrate to a diet high in calcium carbonate, could be produced equally by supplements of copper, manganese or cobalt salts, or by an Analar preparation of iron.

1. METHODS

(i) Construction of diets

As this experiment was in effect an extension of Experiment 2, it was desirable to reproduce as far as possible the same set of conditions. Accordingly, Diet 79 was again employed as the basal diet, and Diet 81 as the high calcium carbonate control diet. The third control diet (consisting of Diet 81 plus 10 p.p.m. of Fe as ferric citrate) was identical with that used in Experiment 2, and was known as Diet 90. Four new diets were constructed by adding to Diet 81 traces of ferrous sulphate, cupric sulphate, manganous sulphate and cobaltous sulphate respectively, all supplements being of an Analar standard of purity.

The quantity of ferrous sulphate ($FeSO_4.7H_2O$) employed was 5.23 mg. per 107 g. of Diet 81. This amounted to a supplement of 10 p.p.m. of Fe, so that the new diet was thus similar to Diet 90 in iron content - or, at least, in its reputed iron content, for the ferric citrate was not quite pure. As has been indicated, the ferric citrate supplement used in Experiment 2 had apparently contributed about 0.15 mg. of copper (as Cu) to each 100 g. of diet (*i.e.* 1.5 p.p.m.). The supplement of copper sulphate in the present experiment was therefore designed to supply at least this amount of copper; a level of 2 p.p.m. was chosen, and was given by 0.82 mg. of $CuSO_4.5H_2O$ per 107 g.

of Diet 81. In the absence of more exact knowledge concerning the requirement of the mouse for manganese, a similar level of supplementation (2 p.p.m.) was used with the manganese salt, and was given by 0.85 mg. of $MnSO_4.4H_2O$ per 107 g. of Diet 81.

The daily requirement for cobalt has been discussed and seen to be very small. From the information available, it was considered that, even allowing for possible variation between species, cobalt supplementation at the rate of 2 p.p.m. of Co should provide a more than adequate intake; and that this, while probably above the minimum polycythaemia-producing level, would nevertheless be within the limits of the toxic dose. This level was accordingly selected, and was provided by 1.00 mg. of $CoSO_4.7H_2O$ per 107 g. of Diet 81.

(ii) Design (see also Technical Appendix)

The experiment called for the comparison of each of four new diets against each of three controls, and of each of the three controls against the other two controls. On the advice of Mr. M. H. Quenouille, the fifty-six mating pairs of mice (eight on each diet) were distributed within a randomised block. The female mice comprised fourteen litters of four animals each, and each doe was allotted her position in the block according to (a) her litter and number, and (b) the result of a random selection of cards bearing diet-numbers. The arrangement resulting from this procedure can be seen in Text Table 33.

(iii) Procedure and Technical Methods (see also Technical Appendix)

The procedure and methods used were similar to those described for Experiment 2, except that the dams were examined on the day that their first litters weaned, only one litter per dam being bred. Serum was retained only from weanlings, and used for turbidity measurements. Organ weights were not recorded, and no histological examinations were made.

In view of the observation in both Experiments 1 and 2 that there were

TEXT TABLE 33.

Exp. 3. Plan of diets and design

Litter	- (79)	$CaCO_3$ plus					
		- (81)	Fe Cit. (90)	Fe SO_4	Cu SO_4	Mn SO_4	Co SO_4
A	4	3	1		2		
B	3		1		4		2
C	2		3	1	4		
D	2	4				3	1
E	3	4		1		2	
F		4	2			1	3
G		1	2	3	4		
H				4	2	1	3
J		4		2		3	1
K	1		3		4		2
L	2	4			3	1	
M		1		3	4	2	
N	2		4	3			1
P			1	2		4	3

In each litter, the numbers 1 to 4 represent the four individuals in decreasing order of weight.

gross differences in the quantity and character of the subcutaneous fat in weanlings, and that these differences appeared to be associated with diet, it was decided to make systematic examinations of this feature. The skin was therefore removed from each weanling and dam shortly after death, and the quantity of subcutaneous fat present in the dorsal region of the trunk was assessed visually; for this purpose an arbitrary scale was employed, ranging from 0 to 8 'units' of fat.

The sires were removed from the mating cage as soon as their mates were seen to be pregnant, but were kept separately and fed on their experimental diet until the 42nd day after mating. After being weighed, they were destroyed with ether and their dorsal fat was assessed as described above. During the course of the experiment, it was decided to employ the colorimetric method of haemoglobin estimation, which had just been developed (see p. 251), to make readings of blood haemoglobin concentration on these animals before discarding them; this was therefore done with all but a few sires which had been previously removed from the experiment.

2. RESULTS AND DISCUSSION.

The results of the experiment are presented in summarised form in Text Table 34.

Five of the fifty-six mated pairs failed to conceive; three of these were on Diets 79 and 90, the two control diets expected to be satisfactory. There was no suggestion of a litter effect, as none of the five does were littermates.

These infertile matings naturally affected the total numbers of young born; and while the mean number born per litter certainly showed some variation between diets, this variation was no greater than the variation between litters (Text

TEXT TABLE 34.

Exp. 3. Summary of results.

Reproduction.			$CaCO_3$ plus				
	- (79)	- (81)	Fe Cit. (90)	$FeSO_4$	$CuSO_4$	$MnSO_4$	$CoSO_4$
1. Mated pairs (Total No.)	8	8	8	8	8	8	8
2. Litters born (Total No.)	7	8	6	8	8	6	8
3. Young born (Total No.)	65	60	49	66	64	57	66
4. Young born/litter (No.)	9.3	7.5	8.2	8.25	8.0	9.5	8.25
5. Litters weaned (Total No.)	4	5	4	3	6	3	4
6. Young weaned (Total No.)	32	28	24	14	23	10	16
7. Deaths (Total No.)	33	32	25	52	41	47	50
8. Survival rate (%)	49.2	46.7	49.4	21.2	36.0	17.5	24.2
9. Total wt. of young weaned (g.)	243.6	205.4	167.8	103.8	144.5	55.7	113.3
10. Mean wt. weaned/litter weaned (g.)	60.8	41.1	42.0	36.1	24.1	18.6	28.3
11. Mean wt./weanling (g.)	7.6	7.3	7.0	7.7	6.3	5.6	7.1
12. Mean 'mating period' (days)	22.4	23.0	20.0	21.3	22.0	20.3	22.3
Dams. (Means)							
13. Wt. when placed on diet (g.)	23.7	22.1	23.1	24.4	21.9	23.0	23.9
14. Final wt. (g.)	30.9	27.0	28.3	30.4	30.1	29.8	29.6
15. Gain in wt. (g.)	7.2	4.9	5.2	6.0	8.2	6.8	5.7
16. Hb (g./100 ml.)	12.3	11.2	12.1	12.0	11.0	10.3	13.2
17. R.B.C. (mill./cu.mm.)	9.7	9.9	10.5	10.1	9.8	10.2	11.6
18. P.C.V. (%)	42	40	43	42	39	38	46
19. M.C.H. ($\mu\mu$g.)	12.7	11.6	11.4	11.9	11.1	9.6	11.3
20. M.C.H.C. (%)	29.1	28.7	28.1	28.7	28.7	25.6	28.6
21. M.C.V. (cμ)	43.6	40.3	40.6	40.2	38.5	37.7	39.4
22. Dorsal fat (units)	4.4	3.9	3.5	4.5	5.1	4.0	3.1

(continued)

TEXT TABLE 34.

Exp. 3. Summary of results.

Sires. (Means)	- (79)	$CaCO_3$ plus - (81)	Fe Cit. (90)	$FeSO_4$	$CuSO_4$	$MnSO_4$	$CoSO_4$
23. Mating wt. (g.)	28.9	28.3	29.6	29.1	28.1	28.6	28.6
24. Final wt. (g.)	31.2	32.0	33.6	35.9	30.8	32.2	34.0
25. Gain in wt. (g.)	2.3	3.7	4.0	6.8	2.7	3.6	5.4
26. Hb (g./100 ml.)	13.8	10.7	14.9	15.3	11.5	12.9	13.7
27. Dorsal fat (units)	4.0	3.5	4.0	4.9	3.8	4.4	5.0
Litters. (Means)							
28. Hb (g./100 ml.)	4.4	3.2	3.5	3.4	3.5	2.5	3.9
29. R.B.C. (mill./cu.mm.)	4.3	3.9	3.9	3.9	4.1	3.4	4.3
30. P.C.V. (%)	19	15	15	15	16	11	20
31. M.C.H. (μμg.)	10.2	8.2	9.1	8.9	8.4	8.0	8.3
32. M.C.H.C. (%)	22.9	21.3	23.7	22.4	22.0	22.4	19.2
33. M.C.V. (cμ)	44.4	38.5	38.5	39.6	38.5	35.9	43.3
34. Dorsal fat (units)	4.0	4.0	4.5	3.3	4.0	4.0	4.3

TEXT TABLE 35.

Exp. 3. Number born

Litter	-	$CaCO_3$ plus						Mean
		-	Fe Cit.	$FeSO_4$	Cu	Mn	Co	
A	8	9	7		10			8.5
B	12		-		10		11	11.0
C	-		10	11	9			10.0
D	9	10				7	8	8.5
E	10	8		8		12		9.5
F		4	6			9	8	6.8
G		6	-	4	7			5.7
H				10	4	-	10	8.0
J		6		11		-	4	7.0
K	10		8		6		8	8.0
L	7	8			8	8		7.8
M		9		7	10	9		8.8
N	9		9	5			8	7.8
P			9	10		12	9	10.0
Mean	9.3	7.5	8.2	8.3	8.0	9.5	8.3	8.4

- = no litter born.

Table 35). No conclusions can therefore be drawn from these results; they merely confirm the view, expressed previously, that very large numbers of animals must be employed to establish significant differences in an attribute with such a high degree of variability. The 'mating period' (i.e. the length of time between mating and parturition) was also very variable, and there were no clear-cut dietary effects (Text Table 34, line 12).

The rearing performance was extremely poor on all diets. As many as 22 of the 51 litters were not reared, even in part, and among the remainder there was also a high proportion of deaths. Indeed, on no diet did the overall survival rate reach 50%, and in the four new experimental diets it was particularly disastrous. This state of affairs, which virtually ruined the entire experiment, rendered accurate comparisons between diet-groups out of the question.

The death-rate was so high, indeed, as to suggest that some extraneous factor had been involved, there being no evidence that diet had played any part. There was, however, a very marked littermate effect; in fact, complete failure to rear litters born was confined to those dams from litters G-P. These animals were younger than those from litters A-F, but they had all been mated at a similar chronological age. The fact that the older mice had been more successful might have meant simply that they were more resistant to whatever was the cause of the failure to rear litters, but it could also have meant that some harmful factor had been suddenly introduced into the environment. So far as could be determined, however, conditions had not changed in any way. In particular, the diets were still being prepared from the original batches of foodstuffs, and the room temperature had remained steady. There remained the possibility of an infection having been introduced; indeed, the sudden onset of the high death-rate made such an explanation appear not unlikely. On the other hand, it was notable that mice kept under the same conditions but on stock diet remained unaffected. Besides causing the deaths of infant mice, an infection might also have had the property of causing an anaemia of sepsis (see p.38) in surviving weanlings and possibly also in the adults. Any beneficial effects of the dietary supplements would in that case be nullified or minimised.

Most of the deaths in the young mice occurred between 9 and 15 days of age; in most instances no symptoms were observed and the animals had been suckling their dams until within a few hours of death, but in some cases the infants were obviously ill for a day or two before succumbing. Post-mortem examination revealed no fresh abnormalities; the findings were generally similar to those described under Experiment 2, but often the

sucklings were well-grown and the abnormalities not severe. There was no evidence of septicaemia or toxaemia. Bacteriological examination of the organs of the dead animals proved entirely negative; cell-free filtrates prepared from macerated liver, spleen, kidneys, brain and intestinal contents were administered, both orally and by intraperitoneal injection, to healthy unweaned mice (bred on stock diet) varying in age from 4 to 15 days, and also to adult female mice suckling litters, but proved to be harmless in every case.

The sires all remained apparently healthy, and so did the dams that failed to rear their litters. These dams (indicated by an asterisk in Text Table 37) were examined on the day that their last surviving young died, and - although of course they had not been exposed to the stress of rearing a litter - their average blood haemoglobin concentration was found to be very similar to the average for the successful group (12.0 and 11.6 g./100 ml. respectively). When examined post-mortem they showed no abnormalities. The sires and dams thus appeared to have been unaffected, and the cause of the high death-rate in the infant mice remains obscure.

Under such circumstances, statistical analysis of the litter data was impossible, and the making of inferences based on inspection of the means of small unequal groups might be very misleading. The only growth attribute on which any reliance at all might be placed was the mean weight per weanling, since this attribute deals only with survivors. However, even this might well have been influenced by the number of deaths in the litter, and it would be unjust to regard the mean weight of, say, two weanlings surviving from a litter of ten as being comparable with the mean weight of nine survivors from another litter of ten. It must also be remembered that although the young mice concerned had survived, their growth might have been affected by the same factor which had caused the deaths of their fellows. While it is tempting, therefore, to point out that weanlings bred on the copper and manganese diets were lighter than the others (Text Table 34, line 11), it is probably wiser to consider the whole of the reproduction and growth trial as void.

All other aspects of the litter data were also affected by the high death-rate. The haemoglobin values of the weanlings were uniformly poor (Text Table 36), and the differences between the means, even of the control

TEXT TABLE 36.

Exp. 3. Litters. Hb (g./100 ml.)

Litter	-	$CaCO_3$ plus					
		-	Fe Cit.	$FeSO_4$	Cu	Mn	Co
A	2.29	2.22	4.59		3.40		
B	5.33		(-)		4.00		5.18
C	(-)		2.59	4.00	1.78		
D	4.51	2.52				1.92	2.22
E	5.40	3.26		4.07		2.96	
F		5.03	3.40			2.52	2.96
G		-	(-)	-	-		
H				-	5.62	(-)	-
J		-		2.22		(-)	-
K	-		3.55		3.26		-
L	-	2.96			2.81	-	
M		-		-	-	-	
N	-		-	-			-
P			-	-		-	5.18
Mean	4.38	3.20	3.53	3.43	3.48	2.47	3.89

(-) = no litter born.

\- = no litter reared.

diets, much smaller than had been hoped for. In view of what has been said above concerning the unrepresentative nature of the surviving weanlings, it seems best to consider these results also as void. The same must be said of the other litter haematological attributes (Text Table 34, lines 29-33), although it is justifiable to infer from them that the anaemia was microcytic and hypochromic in all diet-groups.

While the haematological results in the dams (Text Table 37 and Text Table 34, lines 16-21) were more complete, the fact that almost half of these animals had failed to rear their litters introduced an unfortunate complication. The stress of reproduction and lactation is an important factor in exposing dietary deficiencies, and without them differences may not be so marked. The five does which failed to conceive at all were disregarded, but the others have been included in the means. Whether some of these animals had been affected by the same agent as caused the death-rate in the infants (it may even have acted through the medium of the dams, such as by interrupting lactation) is a question that cannot be answered. However, the fact that the Hb and P.C.V. results with the three control diets showed the expected relationship, although differences were small, suggests that the results may have been reliable. The diet containing ferrous sulphate gave results very similar to those obtained with Diet 90. The highest figures were given by the cobalt-rich diet, which appeared to induce a polycythaemia, but the manganese-rich diet especially, and also the copper-rich diet, gave poor results no better than Diet 81. With the erythrocytic indices, differences were too small to be important, although the low Hb values with the manganese and copper diets appeared to be associated with microcytic hypochromic red cells.

The results given by the sires, although not complete, are interesting. Adult males would not have been expected to exhibit such big haematological

TEXT TABLE 37.

Exp. 3. Dams. Hb (g./100 ml.)

Age (days younger than litter A)	Litter	-	$CaCO_3$ plus					
			-	Fe Cit.	$FeSO_4$	Cu	Mn	Co
-	A	9.92	9.18	11.84		10.21		
0	B	15.10		-		12.14		15.39
0	C	-		8.88	10.36	10.95		
7	D	11.25	10.36				10.36	12.14
7	E	12.28	11.84		11.54		10.95	
7	F		14.50	11.84			8.29	8.58
7	G		9.18x	-	11.99x	5.62x		
8	H				15.39x	12.43	-	13.62x
10	J		7.70x		10.06		-	13.62x
10	K	13.47x		12.28		13.32		13.32x
13	L	12.43x	13.17			12.73	12.73	
13	M		13.62x		12.28x	10.36x	11.54x	
18	N	11.84x		15.39x	13.02x			15.10x
18	P			12.14x	11.25x		7.99x	13.47
	Mean	12.33	11.19	12.06	11.99	10.97	10.31	13.16

- = no litter born.

x = dam failed to rear litter.

differences as the dams, as they were not subjected to stress; but in fact their Hb means showed larger differences (Text Table 34, line 26). Further, although the results with these animals were quite independent of the results with the dams, they followed an essentially similar pattern, high values being recorded from Diets 79 and 90 and from the iron- and cobalt-rich diets, and low values from Diet 81 and from the copper- and manganese-rich diets.

It must be remembered that none of these effects can be considered conclusive, as their statistical significance was not known. Further, variability within diet-groups was in all cases fairly high. However, they are at least suggestive, particularly since the results with dams and sires were independent but still similar, and they indicate that a repetition of the experiment might provide more positive results.

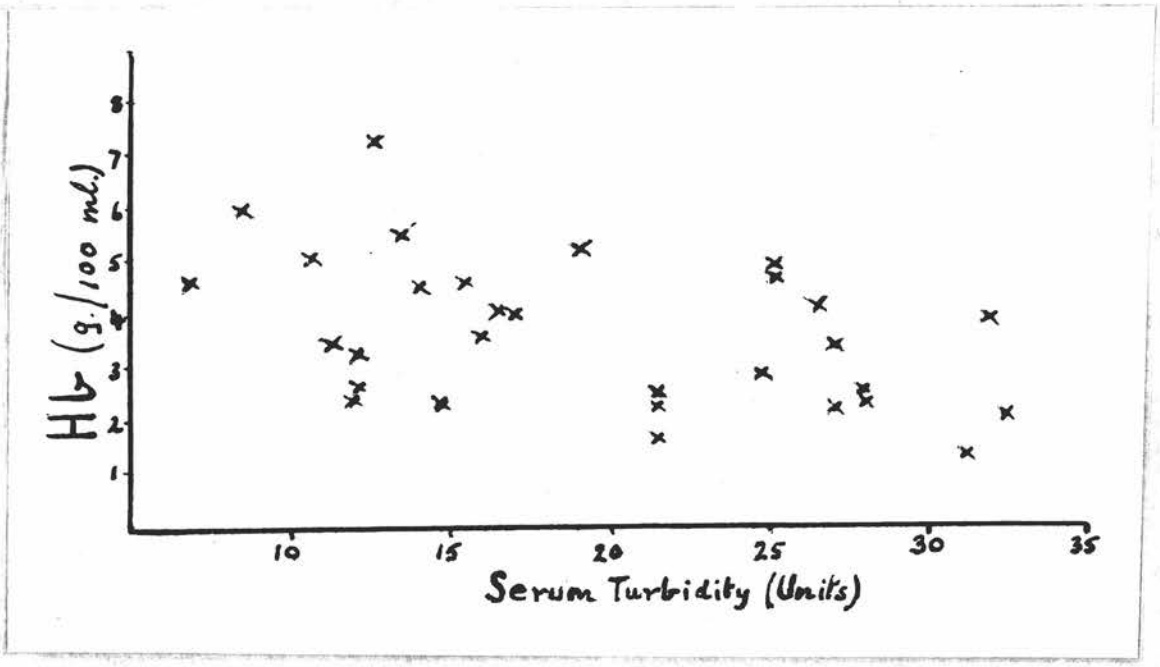
The differences in final weight and in weight gain, especially with the sires, are too small to be considered important. Indeed, large differences could not be expected, since the animals were almost fully grown when put on the diets. However, one conclusion emerges - in no case was the level of supplement high enough to be toxic and cause loss of weight; this indicates that similar levels could therefore be used again with confidence.

The experiment also succeeded in showing that, although it had been previously suspected in weanlings, there was no close inverse relationship between the quantity of dorsal subcutaneous fat and the haemoglobin concentration in adult mice. As with all other litter attributes, no conclusion regarding dorsal fat could be reached with the weanlings in this experiment.

The serum retained from the weanlings in this experiment was used to test a method for the measurement of serum turbidity. The readings obtained are shown graphically in Fig. 30. Although somewhat variable, they point to the probability of an inverse relationship between serum turbidity (mainly due to fat) and haemoglobin concentration, thereby confirming the observations reported

FIGURE 30

Exp. 3. Litters. Serum turbidity and blood haemoglobin concentration



in Experiments 1 and 2.

3. SUMMARY

1. The experiment was carried out to determine whether trace supplements of copper, manganese or cobalt or a supplement of Analar ferrous sulphate, added to Diet 81, would improve reproduction performance and blood values in the same way as had a preparation of ferric citrate in Experiment 2.

2. Five of the 56 mated pairs failed to conceive, but there were no littermate effects.

3. 'Number born' and 'number born per litter' were too variable to warrant conclusions being drawn.

4. Differences in 'mating period' were not clear-cut.

5. Rearing performance was extremely poor on all diets. Possibly this was due to an extraneous factor, but the cause remains obscure.

6. Statistical analysis of the litter data was consequently impossible. The reproduction trial is best considered void. All surviving weanlings were anaemic, the anaemia being microcytic and hypochromic, but dietary effects could not be recognised.

7. The anaemia seen in some dams was microcytic and hypochromic. Both dams and sires showed the same pattern of results with Hb, viz: Diets 79 and 90 and the iron- and cobalt-rich diets, high; Diet 81 and the copper- and manganese-rich diets, low.

8. Differences between diet-group means in final weight and in weight gain were small; but no supplement caused loss of weight.

9. There was no obvious relationship between haemoglobin concentration and dorsal subcutaneous fat in the adult mice.

10. There may have been an inverse relationship in weanlings between serum turbidity and haemoglobin concentration.

In view of the unsatisfactory results of Experiment 3, it was clearly desirable to obtain further information on the effects of the supplements, and for this purpose a further experiment was designed and carried out.

EXPERIMENT 4

1. EXPERIMENTAL PLANS

I decided not to include manganese in this experiment, but rather to concentrate on those elements - iron, copper and cobalt - which might have had some effect on haemoglobin production. I also decided to revise the design, so that the effects of the copper and cobalt supplements might be determined in the presence as well as in the absence of added iron. This arrangement more closely simulated the situation existing in Experiment 2, in which an impure preparation of iron had been used, and any contaminating copper or cobalt had therefore existed in the presence of iron.

The chief advantage of these changes, however, was that they permitted the use of a factorial design; not only could this give more information than a randomised block, and give it more accurately, but it also preserved economy in the numbers of experimental animals required.

As will be seen in the next sub-section, litters of only three breeding females would have sufficed for this purpose. But as a sufficiency of litters of four were available, I decided to utilise them to the full by testing one

further supplement, namely powdered dry thyroid gland. The reasons for the inclusion of this substance must now be explained.

The lipotropic action on the liver and blood serum possessed by certain endocrine glands, notably the thyroid gland, has already been discussed in some detail (pp. 101 and 113). These properties were interesting in view of the fact that fatty livers and hyperlipaemia had been seen in Experiment 2 associated with the calcium carbonate supplement. It was even more interesting to recall that evidence existed (see below) to suggest that the administration of calcium salts induced a hypothyroid state, and consequently it appeared quite possible that the deleterious effects of the calcium carbonate supplement could have been mediated, at least to some extent, through the thyroid gland. The fact that the thyroid gland may affect both erythropoiesis and reproduction (see below) was additional *prima facie* evidence supporting this possibility. Before proceeding with a description of the experiment, the evidence for these statements will now be presented.

The thyroid gland and dietary calcium salts

Observations have been on record for well over one hundred years that the incidence of goitre in the human subject is associated with particular types of soil, especially those rich in calcium. The geological formations most commonly linked with the disease are limestone (including dolomite or magnesian limestone) and calcareous rocks; but there is no invariable rule, and it has been suggested that the absolute iodine content of the soil may be by far the most important factor.

There is also much evidence that a definite relationship exists between the incidence of goitre and the calcium content of drinking water; Wilms (1910) and Répin (1911) both reported that water lost its goitrogenic activity after precipitation of its calcium by boiling; and Murray, Ryle, Simpson and Wilson (1948), in an extensive review of the literature, have quoted evidence to show that the incidence of goitre is greater in 'hard water' than in 'soft water' areas in Great Britain, even although the iodine content of the former is at least as great as that of the latter.

Experimental evidence also indicates that diets high in calcium salts, especially if at the same time they are low in iodine, induce or intensify thyroid hyperplasia in experimental animals (Tanabe, 1924-5; Abelin, 1928; Hellwig, 1931, 1934, 1935; Thompson, 1932, 1933a, b; Klein, 1933-4;

Sampson and Putzki, 1952). It must be pointed out, however, that some authors believe that calcium chloride, although goitrogenic, owes its activity chiefly to its chloride ion (Hibbard, 1933; Sharpless and Anthony, 1943); others, notably Levine and his colleagues (Levine, Remington and von Kolnitz, 1933; Remington and Levine, 1936; Remington, Coulson and Levine, 1936), have stated that the level of dietary calcium and the Ca:P ratio are of only secondary importance to the iodine level of the diet.

The explanation of some of these apparently conflicting observations may lie in the fact, shown by Thompson (1933a, b, 1936), that a high calcium intake will reduce the level of blood iodine, although goitre will not supervene if the iodine intake is maintained at a high enough level. Simpson (1947) explained this effect of calcium on blood iodine by showing that as little as 0.5% of calcium added to the diet of rats led to a decreased concentration of iodine in the thyroid gland and an increase in urinary iodine. It is also interesting to note that the metabolism of calcium is affected by the thyroid gland, its excretion being augmented in hyperthyroidism and reduced in hypothyroidism (Aub, Bauer, Heath and Roper, 1929; Puppel and Curtis, 1936; Robertson, 1942; Owen, 1948). It seems that dietary calcium becomes goitrogenic only after it has been absorbed, since high-calcium diets are harmless in the absence of vitamin D (Sharpless, Sabol, Anthony and Argetsinger, 1943).

The balance of the evidence at present available clearly indicates that dietary calcium after absorption can be goitrogenic.

The thyroid gland and erythropoiesis

Kishi (1904) demonstrated that anaemia followed complete thyroidectomy in the cat, dog and ape, and since then there have been numerous reports to the same effect dealing with a wide variety of species. Among these are the observations of Tatum (1912, 1913), Furuya (1924), Kunde (1926), Kunde and Carlson (1927), Kunde, Green and Burns (1932), Sharpe and Bisgard (1936) and Bisgard and Sharpe (1937), all of which were made on rabbits. In the human subject, anaemia following thyroidectomy has been reported by Stern and Altschule (1936), Limarzi, Keeton and Seed (1937), Jones (1940), Paul, Limarzi and Seed (1943), Burns (1944) and Wilson (1944). Careful experimental observations on rats by Crafts (1941a, 1946b) and Gordon, Kadow, Finkelstein and Charipper (1946) have led to the same conclusion.

Anaemia seems to have been associated with hypothyroidism for the first time by Charcot (1881). A few years later, Horsley (1885) made a similar observation, and the London Clinical Society, in its Report on Myxoedema (1888), also stated that this disease was accompanied by anaemia and changes in the blood picture. This finding has since been confirmed very many times, in both spontaneous myxoedema and in other cases of clinical or experimental hypothyroidism (MacCarrison, 1917; Minot, 1921; Emery, 1923; Mackenzie, 1926; Stone 1928; Lawrence and Rowe, 1928; Adams and Sheeket, 1929; Hoskins and Sleeper, 1929; Lisser and Anderson, 1931; Hoskins and Jellinek, 1932; Lerman and

Means, 1932; Holbøll, 1936; Sharpe and Bisgard, 1937; Sharpe, 1937; Bomford, 1938; Meyers, Price, Mack, Foster and Sharp, 1938; Jones, 1939; Jones, 1940; Gordon et al., 1946; Axelrod and Berman, 1950).

In the great majority of these reports, where details were given the anaemia was moderate in degree, macrocytic, and hyperchromic or sometimes normochromic in type; but microcytic, hypochromic anaemia has also been recorded in association with hypothyroidism (Sharpe, 1937; Bomford, 1938; Crafts, 1946b). Where the bone marrow was examined, the reports invariably suggest that erythropoiesis had become impaired, the marrow having become hypoplastic.

Hypothyroidism has also been associated with a high incidence of achlorhydria and disturbances in gastric secretion (Lerman and Means, 1932; Bomford, 1938; Wintrobe, 1942; Sun, Shay, Siplet and Gruenstein, 1952). While a number of such cases in the human subject will respond to liver extract, suggesting deficiency of Castle's intrinsic factor (Means, Lerman and Castle, 1931), this is not the rule. The hypoplastic marrows so commonly reported in this condition would not accord with such an explanation, while there are also other differences in the syndromes of hypothyroid anaemia and pernicious anaemia (Bomford, 1936). Most authorities ascribe the anaemia of hypothyroidism, with its marrow hyperplasia, to atrophy of the erythron (the word suggested by Boycott (1929-30) to include the whole of the blood and the blood-forming organs); this being a physiological compensation for the lowered metabolic rate and the diminished need of the tissues for oxygen (Minot, 1921; Hoskins and Jellinek, 1932; Stern and Altschule, 1936; Bomford, 1936, 1938; Wintrobe, 1946). The anaemia is therefore akin to that found in animals exposed to high oxygen tension (Bomford, 1938). But, as Whitby and Britton (1950) point out, this view does not explain why the red cells are macrocytic in hypothyroidism. According to Haden (1937), macrocytosis usually results from failure to complete the formation of the nature erythrocyte*, and he has suggested that in the anaemia of hypothyroidism the marrow is unable to utilise an "erythrocyte maturing factor" in the absence of thyroid hormone. This suggestion anticipated the report of Christensen and Plum (1947) that the thyroid gland produces substances of importance to the activation of the reticulocyte-ripening substances (see p. 34).

Bomford (1936, 1938) offered a convincing explanation for the conflicting reports on the type of anaemia found in hypothyroidism. He believed that uncomplicated hypothyroidism leads to a contraction of the erythron and the appearance of the typical macrocytic hyperchromic anaemia, curable only by the administration of thyroid hormone. Occasionally, in the human subject, this anaemia is complicated by Addisonian pernicious anaemia, so that both thyroid hormone and liver extract are required to overcome it. The third type, hypochromic anaemia, develops when there is a superimposed iron deficiency. This

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* This is quite a reasonable opinion in view of suggestions by Bürker (1922), Mills (1925) and Emmons (1927-8) that haemoglobin has a surface distribution within the corpuscle; for clearly if there is a limit on the number of cells which can be formed, but no shortage of haemoglobin, blood containing large cells will have a greater oxygen-carrying capacity than blood containing small ones.

may well result from an interference with iron absorption due to the lowered gastric acidity associated with hypothyroidism.

The same author (Bomford, 1938) showed that this type of anaemia, if treated with thyroid extract alone, became more microcytic although the anaemia did not improve, whereas if iron were given alone there was some improvement, the cells becoming distinctly macrocytic and hyperchromic; this condition could then be completely corrected by the additional administration of thyroid extract. Similar findings were reported by Crafts (1946b) with hypophysectomised as well as with thyroidectomised rats; this worker found iron and copper more effective than iron alone. His work also suggested that hypothyroidism (induced by hypophysectomy) may interfere with iron metabolism, particularly in relation to the synthesis of haemoglobin. Meyers, Price, Mack, Foster and Sharp (1938) had earlier speculated that the anaemia of hypothyroidism in man might be due to a diminution in the rate of iron metabolism. Crafts and Walker (1947b) also observed a failure on the part of hypophysectomised rats to utilise iron for haemoglobin synthesis. A recent paper by Lederer (1950) again suggests a relationship between iron and thyroid hormone, since three hyperthyroid but iron-deficient women showed no elevation in B.M.R. until the deficiency of iron was corrected.

There is much less unanimity on the effects of hyperthyroidism on the blood picture and on erythropoiesis. The majority opinion is that - initially, at least - the red cell count tends to be higher in most cases of experimental and natural hyperthyroidism, while increased cellularity of the marrow has also been frequently recorded (Kocher, 1908; Parhon, 1911; Lim, Sarkar and Brown, 1922; Adams and Sheoket, 1929; Jedlowski, 1931; Axelrod and Berman, 1950). On the other hand, in an extensive series of cases of clinical hyperthyroidism in the human subject, Jackson (1931) found no important change in the red cell picture, while some reports indicate that hyperthyroidism, particularly if prolonged, can lead to anaemia with a hypoplastic marrow - though the initial effect may be a polycythaemia with a hyperplastic marrow (Mackenzie, 1926; Kunde et al., 1931-2; Hoskins and Jellinek, 1932; McCullagh and Dunlap, 1932; Hertz and Lerman, 1932; Gottlieb, 1934; Latta and Benner, 1934; Jones, 1940; Burns, 1944; Gessler, 1946; Sikkema, Thewlis and Meyer, 1946). In some cases at least the red cells are hypochromic (Földes, 1924; Kunde et al., 1931-2; Gessler, 1946) but some hold that the response is non-specific and linked with the demand of the tissues for oxygen (Hoskins and Sleeper, 1929; Hoskins and Jellinek, 1932). In this way it may resemble the haemopoietic response to low oxygen tension (see p. 32).

Indeed, the thyroid hormone may be essential for this response, as Mannsfeld (1913) and Askanazy (1930) showed that it was not given by animals which had been previously thyroidectomised. This point has been disputed by Meyer, Thewlis and Rusch (1940), but the same group of workers (Stewart, Greep and Meyer, 1935; Meyer, Stewart, Thewlis and Rusch, 1937) have shown that hypophysectomy will abolish the reticulocytosis and bone marrow hyperplasia characteristic of exposure to low oxygen tensions, provided that an interval of some 25 or more days is allowed to elapse after the operation. Since the hypophysis probably exerts its effects on haemopoiesis partly, at least, through its influence on the thyroid gland (Crafts, 1946b; Meyer, Thewlis and Rusch, 1940; Vollmer et al., 1942), the interval may be required for the elimination of the thyrotropic hormone

already circulating in the blood. Further evidence of the part played by the thyroid hormone, in inducing an erythropoietic response to oxygen want, comes from Furuya (1924), who found that thyroidectomised rabbits gave only a slow response to the stimulus provided by exsanguination. Gordon et al. (1946) confirmed this in rats, and found that the injection of thyroxin accelerated the regeneration of red cells and raised the blood haemoglobin concentration. It is interesting to note that these workers also observed that the administration of cobalt gave a similar result, and that the effects of thyroxin and cobalt were additive.

The thyroid gland and reproduction

There is ample evidence that the reproductive processes are to some extent regulated by the thyroid gland, especially in the female.

In the human subject, embryonic death, stillbirth and premature birth have been associated with hypothyroidism (King and Herring, 1939; Kemp, 1939). Experimental work with laboratory animals has shown that deficiency of the thyroid hormone may lead to irregularities in the oestrous cycle, although the ovaries may be morphologically normal (Laqueur and Emge, 1941; Williams, Phelps and Burch, 1941). Soliman and Reineke (1952) found that the ovaries of hypothyroid mice were packed with follicles but that corpora lutea were not formed, and they also quote other reports which indicate that the thyroid secretion is concerned in the reproductive process. Similar evidence is cited by Hurst and Turner (1948), who also report their own findings that both hypothyroidism and hyperthyroidism adversely affect gonad function in female mice. A further brief review of the subject has been offered by Albert (1952). In hypothyroid male rats, Hölscher, Böckh and Hardegg (1949) and Maqsood and Reineke (1950) both found that spermatogenesis was reduced or inhibited.

The evidence concerning the effects of the thyroid secretion on lactation, which is reviewed by Hurst and Turner (1948), is confused and conflicting, but these workers conclude that it seems probable that hypothyroidism decreases lactation in the rat.

2. METHODS

(i) Design

The experiment was originally planned to compare an unsupplemented control diet with similar diets containing supplements of copper or cobalt, in the presence of additional iron, calcium carbonate, neither or

both. The addition of a fourth series of diets containing a supplement of desiccated thyroid completed the final plan shown in Text Table 38.

The effects of iron and of calcium carbonate were of secondary importance in this experiment. Consequently, Mr. A. W. Boyne prepared for me a design in which the comparisons between the copper, cobalt, thyroid and control series were all within-litter comparisons, while the remainder were between-litter comparisons. This was achieved by using sixteen litters each of four female mice, and designing the experiment in four replications. Each replication consisted of four litters chosen at random, one litter being allocated to each row in the plan with the individual members of the litter distributed at random among the columns.

(ii) Construction of diets

As has been stated, this experiment was concerned mainly with haematological attributes, and it was therefore desirable to have as a control diet one which would support a high haemoglobin value. For this reason, the basal diet selected was the original modification of the Sherman B diet used as the basal diet in Experiment 1, and was known as Diet 2. This was preferred to Diet 79 because Experiment 1 had shown that the haemoglobin values of animals in its first column (with no added calcium carbonate, e.g. Diet 2) had been equally as high and possibly higher than those in its second column (with 0.5% added calcium carbonate, e.g. Diet 79), and at the same time there had been no differences between its rows (Diet 2 having been in the first row and Diet 79 in the fourth row).

The levels of calcium carbonate and iron supplements were kept similar to those used in previous experiments, being 2 g. of calcium carbonate per 100 g. of basal diet, and 10 p.p.m. of Fe as hydrated ferrous sulphate ('Analar'). The use of these supplements alone or in combination completed column 1 in the

TEXT TABLE 38.

Exp. 4. Plan of Diets.

		Columns			
		- (control)	Copper	Cobalt	Thyroid
Rows	- (Basal)				
	Iron				
	Calcium carbonate				
	Iron plus calcium carbonate				

TEXT TABLE 39.

Exp. 4. Number of litters born

Four mated pairs on each diet

(a) First litters

	-	Cu	Co	Thyroid	Total
-	3	4	4	4[x]	15[x]
Fe	3	3	4	4	14
$CaCO_3$	4	4	4	4	16
Fe + $CaCO_3$	4	4	4	4	16
Total	14	15	16	16[x]	61[x]

[x] One litter devoured by its dam at birth.

(b) Second litters

	-	Cu	Co	Thyroid	Total
-	3	4	4	4	15
Fe	3	3	3	4	13
$CaCO_3$	3	4	4	4	15
Fe + $CaCO_3$	4	4	4	4	16
Total	13	15	15	16	59

plan of the present experiment. As supplements to complete columns 2 and 3, cupric sulphate and cobaltous sulphate were used in the same quantities as in Experiment 3. The desiccated thyroid preparation used as the supplement for column 4 was Pulv. Thyroid Sicc. B.P.;* the dose employed was 0.2% of the basal diet, this quantity having been found to be not severely toxic to mice but yet sufficient to increase their metabolic activity (Simpson, B. W. - personal communication).

Diets were prepared as described in the Technical Appendix.

(iii) Procedure and Technical Methods

The mating procedures were similar to those used in Experiment 2; in addition, records were kept of the initial and final weights of the sires.

Haemoglobin concentration and packed cell volume were estimated on litters and dams at the same times and in the same way as in Experiment 2; red cell counts were omitted. Estimations of haemoglobin concentration were also made on the sires when they were removed from the mating cages.

Frozen sections of the median lobe of the liver were prepared from a representative of each weaning first litter in the thyroid and control columns, and stained for fat with Scharlach R, using haemalum as a counterstain.

Turbidity measurements were made on serum from the litters; blood from all the weanlings in each litter was pooled, and the serum separated from it in the same way as in Experiment 2. In view of the possibility that diabetes mellitus might have developed as a sequel to a high intake of calcium carbonate, the opportunity was also taken to examine the pancreas in animals from diet-

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* It was thought that this substance, which is prepared from thyroid tissue, might have contained iron derived from blood, and spectrographic examination of the material, conducted for me by Dr. R. L. Mitchell, showed that its iron content was 0.030% Fe_2O_3. However, in the dose in which it was fed, it thus increased the iron content of the diet by only 0.42 p.p.m. - a negligible amount from the point of view of this experiment.

groups containing this supplement. Paraffin sections of this organ, stained haemalum and eosin, were therefore prepared from dams and representative weanlings in the calcium carbonate and control diet-groups of the control column.

3. RESULTS

The data were treated in the same way as in Experiment 2, diet-group means being constructed from individual results or - in the case of litter data - from litter means. The diet-group means were then combined into marginal means. A statistical analysis of the data to determine levels of significance of differences was made for me by Mr. A. W. Boyne. Since the experiment was primarily concerned with the effects of the copper, cobalt and thyroid supplements, he fitted missing observations in such a way as to minimise the residual mean square which tested these supplements and their interactions with other supplements. The estimated effects of calcium carbonate and iron, alone or in combination, were consequently less accurate than were those of the other supplements, but in this way the latter were accorded the maximum possible degree of accuracy.

The results of the experiment are summarised in Text Tables 39-62.

(i) Reproduction and Growth

Analysis of the reproduction data was complicated by the fact that three does failed to reproduce at all and a further two failed to bear second litters (Text Table 39), and there is no means of deciding whether these failures were due to chance or were the results of the dietary treatments. However, all five animals were on different diets; two of the three completely infertile does were littermates, but the third completely infertile doe and also the two animals which produced only one litter were not closely related. In

TEXT TABLE 40.

Exp. 4. Mean number born.

(a) First litters - Unadjusted means (per pregnancy).

	-	Cu	Co	Thyroid	Marginal Means
-	9.3	9.8	8.8	8.7	9.1
Fe	8.0	9.0	8.8	11.5	9.5
$CaCO_3$	9.0	8.0	8.5	10.3	8.9
Fe + $CaCO_3$	7.8	8.8	8.5	9.5	8.6
Marginal Means	8.5	8.9	8.6	10.1	9.0

(b) First litters - adjusted mean square roots (per doe).

	-	Cu	Co	Thyroid	Marginal Means
-	2.075	3.145	2.958	2.960	2.784
Fe	2.120	2.248	2.955	3.385	2.677
$CaCO_3$	2.995	2.820	2.912	3.190	2.979
Fe + $CaCO_3$	2.772	2.955	2.910	3.042	2.920
Marginal Means	2.491	2.792	2.934	3.144	2.840

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 0.68	± 0.34
Columns	± 0.46	± 0.23

(continued)

(continued)

TEXT TABLE 40.

Exp. 4. Mean number born.

(c) Second litters - unadjusted means (per pregnancy).

	-	Cu	Co	Thyroid	Marginal Means
-	7.0	7.8	2.8	9.0	6.6
Fe	9.7	8.3	2.3	6.8	6.8
$CaCO_3$	8.7	7.5	8.0	10.0	8.5
Fe + $CaCO_3$	9.5	7.8	6.8	10.8	8.7
Marginal Means	8.8	7.8	5.1	9.1	7.7

(d) Second litters - adjusted mean square roots (per doe).

	-	Cu	Co	Thyroid	Marginal Means
-	1.310	2.745	1.618	2.975	2.162
Fe	2.322	2.165	1.102	2.575	2.041
$CaCO_3$	2.205	2.720	2.755	3.145	2.706
Fe + $CaCO_3$	3.200	2.742	2.502	3.258	2.926
Marginal Means	2.259	2.593	1.994	2.988	2.459

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 0.78	± 0.39
Columns	± 0.58	± 0.29

TEXT TABLE 41.

Exp. 4. Number of litters weaned.

(a) First litters.

	-	Cu	Co	Thyroid	Total
-	1	3	3	2	9
Fe	3	2	1	3	9
$CaCO_3$	3	3	4	1	11
Fe + $CaCO_3$	4	3	3	3	13
Total	11	11	11	9	42

(b) Second litters.

	-	Cu	Co	Thyroid	Total
-	2	4	2	3	11
Fe	3	3	1	2	9
$CaCO_3$	2	3	4	4	13
Fe + $CaCO_3$	4	4	3	4	15
Total	11	14	10	13	48

TEXT TABLE 42.

Exp. 4. Survival rate (percentage of total young born per diet-group surviving until three weeks of age).

(a) First litters.

	-	Cu	Co	Thyroid	Marginal Means
-	4	41	26	11	20
Fe	67	33	23	46	42
$CaCO_3$	47	47	47	10	38
Fe + $CaCO_3$	52	43	53	32	45
Marginal Means	42	41	37	25	36

(b) Second litters.

	-	Cu	Co	Thyroid	Marginal Means
-	48	65	64	50	57
Fe	52	72	57	48	57
$CaCO_3$	65	43	78	67	63
Fe + $CaCO_3$	84	87	89	77	84
Marginal Means	62	67	72	61	65

addition, one further dam on the thyroid only diet (unrelated to any of the infertile animals) devoured her first litter before the young could be counted.

The unadjusted means of the 'Number born' are shown in Text Table 40, (a) and (c); in preparing these means, it was assumed that the infertility in the does was the result of chance and not diet, and so the missing animals were simply given the average value of their diet-group. For the statistical analysis, on the other hand, it was assumed that diet had been responsible for the five does being infertile and a value of zero was therefore given in these cases. Because of the high variability of the data, the statistical analysis was conducted on square roots (Text Table 40, (b) and (d)). It showed that the mean value given by diet-groups receiving thyroid was significantly higher than that of the control groups, this being true of both first and second litters ($\underline{P} < 0.05, < 0.01$ respectively); the analysis also showed that, in second litters, the mean number of young born to dams on diets containing calcium carbonate was significantly ($\underline{P} < 0.05$) higher than the number born to the others. However, as can be seen from Text Table 39, three of the five zero figures were in Column 1 (controls) and three in Row 2 (receiving iron but no calcium carbonate), and consequently the effects of the thyroid and calcium carbonate supplements may have been exaggerated in this analysis; nevertheless, it is noteworthy that similar trends are manifest with the simple means.

Of 61 first litters born, 19 were not reared even in part; and of 59 second litters born, 11 were entirely lost before weaning age (Text Table 41). Even in the remaining litters, especially first litters, there was in many cases a heavy death-rate. The 'Survival Rate' (i.e. percentage of young born reaching weaning age) is shown in Text Table 42. With first litters, differences between the means are unimportant, but with second litters there is a suggestion that the presence of calcium carbonate improved the performance.

Analysis of the 'Mating Period' (i.e. the time between mating and

TEXT TABLE 43.

Exp. 4. Mating period (days).

	-	Cu	Co	Thyroid	Marginal Means
-	21.5	21.0	20.8	23.8	21.8
Fe	18.2	19.8	21.8	35.2	23.8
$CaCO_3$	22.5	21.8	21.8	22.5	22.1
Fe + $CaCO_3$	22.5	21.5	21.5	29.5	23.8
Marginal Means	21.2	21.0	21.4	27.8	22.8

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 6.46	± 3.23
Columns	± 5.04	± 2.52

TEXT TABLE 44.

Exp. 4. Period between litters (days).

	-	Cu	Co	Thyroid	Marginal Means
-	23.2	24.0	26.2	25.5	24.8
Fe	31.8	29.2	29.5	33.5	31.0
$CaCO_3$	26.2	40.8	31.0	37.2	33.8
Fe + $CaCO_3$	42.2	35.8	31.8	45.5	38.8
Marginal Means	30.9	32.4	29.6	35.4	32.1

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 11.97	± 5.98
Columns	± 7.66	± 3.83

TEXT TABLE 45.

Exp. 4. Mean number weaned.

(a) First litters - unadjusted data (per pregnancy).

	-	Cu	Co	Thyroid	Marginal Means
-	0.3	4.0	2.3	1.0	2.1
Fe	5.3	3.0	2.0	5.3	3.9
$CaCO_3$	4.3	3.8	4.0	1.0	3.3
Fe + $CaCO_3$	4.0	3.8	4.5	3.0	3.8
Marginal means	3.6	3.7	3.2	2.7	3.3

(b) First litters - adjusted mean square roots (per doe).

	-	Cu	Co	Thyroid	Marginal Means
-	0.250	1.662	1.162	0.602	0.919
Fe	1.662	1.045	0.708	1.942	1.339
$CaCO_3$	1.722	1.570	1.820	0.500	1.403
Fe + $CaCO_3$	1.958	1.628	1.765	1.412	1.691
Marginal means	1.398	1.476	1.364	1.114	1.338

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 0.91	± 0.46
Columns	± 0.69	± 0.34

(continued)

(continued)

TEXT TABLE 45.

Exp. 4. Mean number weaned.

(c) Second litters - unadjusted data (per pregnancy).

	-	Cu	Co	Thyroid	Marginal Means
-	3.3	5.0	1.8	4.5	3.7
Fe	5.0	6.0	1.3	3.3	3.8
$CaCO_3$	5.7	3.3	6.3	6.8	5.5
Fe + $CaCO_3$	8.0	6.8	6.0	8.3	7.3
Marginal Means	5.7	5.2	4.0	5.7	5.1

(d) Second litters - adjusted mean square roots (per doe).

	-	Cu	Co	Thyroid	Marginal Means
-	0.500	2.240	0.912	1.828	1.370
Fe	1.655	1.802	0.500	1.275	1.308
$CaCO_3$	1.458	1.475	2.458	2.570	1.990
Fe + $CaCO_3$	2.992	2.548	2.098	2.848	2.621
Marginal Means	1.651	2.016	1.492	2.130	1.822

Standard error of difference between:

	Tabular Means	Marginal Means
Rows	± 0.95	± 0.48
Columns	± 0.67	± 0.34

parturition) showed (Text Table 43) that the thyroid supplement increased this period to a significant extent ($P < 0.05$). Although the difference was not significant, it is interesting to note that a similar tendency can be seen in the analysis of 'Period between litters' (Text Table 44). There is also a strong suggestion that the calcium carbonate supplement augmented the interval between the birth of first and second litters ($P \neq 0.05$).

The 'Number Weaned' (Text Table 45) was analysed in the same way as was 'Number Born', and while diet-groups containing calcium carbonate appeared to be superior with second litters ($P < 0.05$), this result was partly attributable to differences in numbers born, while the possible causes of exaggerated effects, which were mentioned in connection with 'Number Born', apply also to 'Number Weaned'.

Weaning weights are shown in Text Tables 46 and 47. Owing to the high variability of the survival rate between individual diet-groups, and also to the zeros resulting from the infertility of five animals, the diet-group totals (Text Table 46) are also very variable and cannot be considered separately. The marginal totals, however, show that the animals receiving thyroid reared the lowest total weight with their first litters; this poor result is emphasised if the marginal totals are adjusted for the infertile does (on the assumption that the infertility was unconnected with diet), i.e. in the attribute 'Mean weight weaned per litter born'. With second litters the performance was much improved in all groups; differences between totals and means were small, although the good performance of Row 4 (calcium carbonate plus iron) deserves notice. The attribute 'Mean weight weaned per litter weaned' (constructed by dividing the marginal totals by the number of litters contributing to them) also gives a similar result; the thyroid column can be seen to have the lowest value with both first and second litters, while the rows containing iron gave the best results.

TEXT TABLE 46.

Exp. 4. Total and mean weights weaned (g.) (tabular totals are for the four animals in each diet-group; marginal totals for the 16 animals in each row or column).

(a) First litters.

	-	Cu	Co	Thyroid	Total	Mean wt. per litter	
						Born	Weaned
-	10.6	123.8	69.9	17.5	221.8	14.8	24.6
Fe	128.0	90.3	91.4	145.8	455.5	32.5	50.6
$CaCO_3$	103.5	101.2	111.2	18.8	334.7	20.9	30.4
Fe + $CaCO_3$	125.4	131.2	147.6	72.9	477.1	30.0	36.7
Total	367.5	446.5	420.1	255.0	1489.1		
ean wt. per litter born	26.3	29.8	26.3	15.9		24.6	
ean wt. per litter weaned	33.4	40.6	38.2	28.3			35.5

(b) Second litters.

	-	Cu	Co	Thyroid	Total	Mean wt. per litter	
						Born	Weaned
-	134.5	180.8	92.4	136.1	543.8	36.3	41.4
Fe	123.4	194.7	51.4	109.0	478.5	36.8	53.2
$CaCO_3$	154.2	116.5	211.7	165.0	647.4	43.2	49.8
Fe + $CaCO_3$	279.7	235.6	233.4	253.5	1002.2	62.6	66.8
Total	691.8	727.6	588.9	663.6	2671.9		
ean wt. per litter born	53.2	48.5	39.3	41.5		45.3	
ean wt. per litter weaned	62.9	52.0	58.9	51.0			55.7

TEXT TABLE 47.

Exp. 4. Mean weight per weanling (g.)

(a) First litters.

	-	Cu	Co	Thyroid	Marginal Means	Total wt. weaned / No. weaned
-	10.6	7.8	7.8	5.8	8.0	7.6
Fe	8.0	10.0	11.4	6.9	9.1	8.4
$CaCO_3$	6.1	6.7	7.0	4.7	6.1	6.4
Fe + $CaCO_3$	7.8	8.7	8.2	6.1	7.4	7.8
Marginal Means	8.1	8.3	8.6	5.9	7.7	
Total wt. weaned / No. weaned	7.4	8.1	8.2	6.4		7.6

(b) Second litters.

	-	Cu	Co	Thyroid	Marginal Means	Total wt. weaned / No. weaned
-	13.5	9.0	13.2	7.6	10.8	9.9
Fe	8.2	10.8	12.9	8.4	10.1	9.6
$CaCO_3$	9.1	9.0	8.5	6.1	8.2	7.9
Fe + $CaCO_3$	8.7	8.7	9.7	7.7	8.7	8.6
Marginal Means	9.9	9.4	11.1	7.5	9.4	
Total wt. weaned / No. weaned	9.3	9.3	9.8	7.3		8.8

It is of importance to consider not only the litter weights but also the weights of the individuals comprising the litters, and by eliminating the influence of the number of weanlings comprising each litter we obtain the attribute 'Mean weight per weanling' (Text Table 47). The most striking result with this attribute is that in both first and second litters the thyroid column is consistently the lightest. It is also notable that the presence of iron increased the mean and the presence of calcium decreased it. As some diet-groups, especially with first litters, contained very few weanlings (whose individual weights were consequently unduly important to the means), overall means were also calculated for columns and rows. These, however, gave a similar if less marked result.

It must be remembered, however, that the nature of the data precluded an accurate statistical assessment of the significance of all these results, and they therefore cannot be accepted as firm conclusions.

The weights of the dams at various stages are shown in Text Tables 48, 49 and 50. Clearly these animals were very even in their initial weights when introduced to the diet and also when mated one week later, but their final weights at the end of the experiment showed notable differences. Final weight was lowered by the thyroid supplement ($P \doteq 0.05$), and there was also a suggestion that the copper supplement had had the same effect. The dams on the calcium carbonate diets were consistently lighter than the others, but the difference did not reach the 5% level of significance.

The mating weights of the sires (Text Table 51) were less uniform than those of the dams, and their final weights (Text Table 52) are therefore less interesting than their weight gains (Text Table 53). The analysis was carried out on three replications only, as there were a number of missing observations, but it showed that sires receiving thyroid gained significantly more weight than those that did not ($P < 0.05$).

TEXT TABLE 48.

Exp. 4. Dams. Weight when placed on diet (g.).

	-	Cu	Co	Thyroid	Marginal Means
-	21.05	20.52	21.40	20.68	20.91
Fe	21.70	21.40	20.20	21.45	21.19
$CaCO_3$	21.20	20.85	20.20	20.90	20.79
Fe + $CaCO_3$	20.00	20.10	20.85	19.40	20.09
Marginal means	20.99	20.72	20.66	20.61	20.74

Standard error of differences between:

	Tabular means	Marginal means
Rows	± 1.739	± 0.870
Columns	± 1.007	± 0.504

TEXT TABLE 49.

Exp. 4. Dams. Mating weight (g.).

	-	Cu	Co	Thyroid	Marginal Means
-	24.55	23.05	24.45	23.42	23.87
Fe	23.30	23.80	22.65	24.65	23.60
$CaCO_3$	24.25	23.20	22.20	24.95	23.65
Fe + $CaCO_3$	24.60	23.95	25.30	24.90	24.69
Marginal means	24.18	23.50	23.65	24.48	23.95

Standard error of differences between:

	Tabular means	Marginal means
Rows	± 3.340	± 1.670
Columns	± 1.723	± 0.862

TEXT TABLE 50.

Exp. 4. Dams. Final weight (g.).

	-	Cu	Co	Thyroid	Marginal Means
-	36.05	32.60	36.82	35.58	35.26
Fe	39.20	33.82	36.70	32.98	35.68
$CaCO_3$	32.45	31.85	35.05	28.65	32.00
Fe + $CaCO_3$	31.98	29.78	33.15	29.85	31.19
Marginal Means	34.92	32.01	35.43	31.76	33.53

Standard error of differences between:

	Tabular means	Marginal means
Rows	± 8.621	± 4.310
Columns	± 3.188	± 1.594

TEXT TABLE 51.

Exp. 4. Sires. Mating weight (g.).

	-	Cu	Co	Thyroid	Marginal Means
-	26.3	26.0	27.3	26.4	26.5
Fe	28.2	28.2	26.3	29.6	28.1
$CaCO_3$	29.9	28.4	25.4	27.0	27.7
Fe + $CaCO_3$	27.7	27.3	29.5	27.6	28.0
Marginal Means	28.0	27.5	27.1	27.7	27.6

TEXT TABLE 52.

Exp. 4. Sires. Final weight (g.) (unadjusted data)

	-	Cu	Co	Thyroid	Marginal Means
-	32.3	31.7	30.8	33.0	32.0
Fe	37.0	34.7	33.0	38.4	35.8
$CaCO_3$	34.6	35.1	31.9	36.7	34.6
Fe + $CaCO_3$	32.1	31.4	32.3	35.2	32.8
Marginal Means	34.0	33.2	32.0	35.8	33.8

TEXT TABLE 53.

Exp. 4. Sires. Gain in weight (g.) (adjusted for missing observations).

	-	Cu	Co	Thyroid	Marginal Means
-	3.60	6.37	3.40	8.10	5.37
Fe	8.63	6.33	7.00	9.03	7.75
$CaCO_3$	6.63	7.13	6.30	8.23	7.08
Fe + $CaCO_3$	4.87	4.10	4.23	9.40	5.65
Marginal Means	5.93	5.98	5.23	8.69	6.46

Standard error of differences between (tabular means = ± 2.118
(marginal means = ± 1.059

(ii) Haematology

Haemoglobin concentration. Diet-group means and marginal means, computed as before, are shown in Text Tables 54-57. A summary of the main effects and interactions is given in Text Table 58.

With the dams (Text Table 54), the expected lowering effect of the calcium carbonate supplement was clearly evident and reached a very high level of significance ($P < 0.001$), and the iron effect, although not large enough to be significant, was in the opposite direction. Consequently, the marginal means in the right-hand column of the Table are in the expected order of relative magnitude.

While the overall variation due to the other supplements was not significant ($P \neq 0.1$), most of it could be attributed to the difference between the cobalt and thyroid columns on the one hand and the control and copper columns on the other ($P < 0.05$). This level of significance is exaggerated since three possible comparisons could have been made and only one has been selected; on the other hand, the same tendency is evident with other classes of animal in this experiment (see Text Table 58 (b)(i)).

With first litters (Text Table 55), the large number of missing litters resulted in only two replications of the design being suitable for statistical analysis; the values of t are consequently high, so that only very large effects reach significant levels. However, marginal means for the rows were in the expected order of relative magnitude, and the lowering effect of calcium carbonate easily exceeded the 5% level of significance. The effect of iron was also strong but just failed to reach this level. None of the other supplements had a significant effect but, as was the case with the dams, most of the variation was due to the high values given by the cobalt and thyroid columns.

The negative effect of calcium carbonate and the positive effect of iron were both significant with second litters ($P < 0.001$, < 0.05 respectively), and

TEXT TABLE 54.

Exp. 4. Hb (g./100 ml.) (Dams). Diet-group and Marginal Means.

	-	Cu	Co	Thyroid	Marginal Means
-	15.66	14.43	14.95	15.61	15.16
Fe	15.76	16.43	14.98	14.77	15.48
$CaCO_3$	8.91	10.73	12.88	11.32	10.97
Fe + $CaCO_3$	10.58	10.29	14.95	14.68	12.62
Marginal Means	12.73	12.96	14.44	14.09	13.56

S.E. of differences between { tabular means ± 1.584; marginal means ± 0.799

t (5% level) { rows = 2.3; columns = 2.0

TEXT TABLE 55.

Exp. 4. Hb (g./100 ml.) (First litters). Diet-group and Marginal Means

	-	Cu	Co	Thyroid	Marginal Means
-	4.59	4.29	6.44	6.66	5.49
Fe	8.88	9.40	9.69	9.92	9.47
$CaCO_3$	1.55	2.59	4.00	2.81	2.74
Fe + $CaCO_3$	4.81	4.29	3.03	6.96	4.23
Marginal Means	4.96	5.15	5.79	6.08	5.49

S.E. of differences between { tabular means { rows = ± 2.516; columns = ± 1.228 } marginal means { rows = ± 1.258; columns = ± 0.622 }

t (5% level) { rows = 3.2; columns = 2.4 }

TEXT TABLE 56.

Exp. 4. Hb (g./100 ml.) (Second litters). Diet-group and Marginal Means

	-	Cu	Co	Thyroid	Marginal Means
-	9.10	6.73	9.55	10.73	9.03
Fe	9.50	10.58	11.72	10.63	10.61
$CaCO_3$	2.99	3.48	4.51	4.14	3.79
Fe + $CaCO_3$	4.44	4.26	5.18	9.29	5.79
Marginal Means	6.51	6.26	7.74	8.70	7.30

S.E. of differences between { tabular means { rows = ±2.241; columns = ±1.513 } marginal means { rows = ±1.120; columns = ±0.756 }

t (5% level) { rows = 2.3; columns = 2.1 }

once again weanlings from the thyroid and cobalt groups had higher values than the others ($P < 0.01$) (Text Table 56). The thyroid supplement was particularly effective. The means given by second litters were all distinctly higher than those of first litters.

Differences between groups were smaller with the sires (Text Table 57), but so were within-group differences. The overall variation due to the calcium carbonate and iron supplements did not reach significance ($0.1 > P > 0.05$), but most of it was due to the lowering effect of the calcium carbonate ($P < 0.05$). The other supplements affected the figures to a significant extent ($P < 0.05$), chiefly owing to the low values given by the copper column and the high values given by the thyroid column.

The main effects have been considered above as though they were unaffected by interactions. In fact, however, as can be seen from Text Table 58(a) and (b)(ii), three interactions were large enough to be significant - namely, cobalt x calcium carbonate with both the dams ($P < 0.01$) and the sires ($P < 0.05$), and thyroid x calcium carbonate with the dams ($P < 0.05$). In each case the interaction was positive in direction. With the dams, cobalt and thyroid had marked raising effects in the presence of calcium carbonate ($P < 0.001$, < 0.01 respectively); but in its absence they had no effect. With the sires, cobalt reduced the value ($P < 0.05$) unless calcium carbonate was also present.

Of the 40 interactions tested, these three were the only ones to reach significance. The size of the cobalt x calcium interaction with the dams signifies that it was almost certainly real; but the other two barely exceeded the 5% level of significance, and it is therefore less certain that they were not chance effects. On the other hand, the interaction noted with the sires' values was the same interaction as was observed with the dams, and was in the same direction; and the thyroid x calcium carbonate interaction is also positive and fairly large with the sires and with second litters (in both cases

TEXT TABLE 57.

Exp. 4. Hb (g./100 ml.) (Sires). Diet-group and Marginal Means.

	-	Cu	Co	Thyroid	Marginal Means
-	14.95	14.21	13.84	15.02	14.50
Fe	15.32	14.98	14.21	15.47	14.99
$CaCO_3$	14.33	12.95	14.28	14.18	13.93
Fe + $CaCO_3$	13.50	13.76	14.65	15.47	14.34
Marginal Means	14.52	13.97	14.24	15.04	14.44

Standard error of difference between { tabular means = ± 0.749; marginal means = ± 0.374

t (5% level) { rows = 2.0; columns = 2.0

TEXT TABLE 58.

Exp. 4. Hb. Effects and Interactions.

(a) ROWS

	Fe	$CaCO_3$	Fe x $CaCO_3$	S.E.±	$\frac{t}{(5\%\ level)}$
Dams	+0.995	-3.528^{xxx}	+0.670	0.556	2.3
Sires	+0.453	-0.610	-0.037	0.265	2.0
1st litters	+2.756	-3.978^{x}	-1.221	0.889	3.2
2nd litters	+1.794^{x}	-5.032^{xxx}	+0.213	0.792	2.3

(b) COLUMNS

(i) Effects

	Cu	Co	Thyroid	S.E.±	$\frac{t}{(5\%\ level)}$
Dams	+0.24	+1.717^{x}	+1.36	0.801	2.0
Sires	-0.55	-0.28	+0.52	0.374	2.0
1st litters	+0.19	+0.83	+1.12	0.616	2.4
2nd litters	-0.25	+1.23	+2.190^{x}	0.756	2.1

	Ca & Thyroid minus Control & Cu	S.E.±	$\frac{t}{(5\%\ level)}$
Dams	+1.42^{x}	0.567	2.0
Sires	Not combined	-	-
1st litters	+0.87	0.434	2.4
2nd litters	+1.84^{xx}	0.533	2.1

(continued)

(continued)

TEXT TABLE 58.

Exp. 4. Hb. Effects and Interactions.

(ii) Interactions

	Cu x Fe	Cu x $CaCO_3$	Co x Fe	Co x $CaCO_3$	Thyroid x Fe	Thyroid x $CaCO_3$	Cu x Fe x $CaCO_3$	Co x Fe x $CaCO_3$	Thyroid x Fe x $CaCO_3$	S.E.±	t (5% level)
Dams	-0.06	+0.52	-0.09	$+2.457^{xx}$	+0.19	$+1.887^{x}$	-1.01	+0.12	+0.67	0.801	2.0
Sires	+0.52	-0.01	+0.30	$+0.833^{x}$	+0.55	+0.40	+0.31	+0.30	+0.52	0.374	2.0
1st litters	-0.19	+0.07	-1.32	-0.50	-0.53	-0.43	-0.59	-0.80	-0.01	0.616	2.4
2nd litters	+0.70	+0.40	+0.25	-0.10	+0.80	+0.96	-1.04	-0.64	+1.05	0.756	2.1

Co x $CaCO_3$	(dams)	Effect of Co in presence of $CaCO_3$ = $+4.166^{xxx} \pm 0.113$ " " " " absence " " = -0.747 ± 0.113	$2.457^{xx} \pm 0.801$
Co x $CaCO_3$	(sires)	Effect of Co in presence of $CaCO_3$ = $+0.555 \pm 0.531$ " " " " absence " " = $-1.110^{x} \pm 0.531$	$0.833^{x} \pm 0.374$
Thyroid x $CaCO_3$	(dams)	Effect of Thyroid in presence of $CaCO_3$ = $+3.256^{xx} \pm 1.132$ " " " " absence " " = -0.518 ± 1.132	$1.887^{x} \pm 0.801$

^{x}Significant ($P < 0.05$)

^{xx}Significant ($P < 0.01$)

^{xxx}Significant ($P < 0.001$).

TEXT TABLE 59.

Exp. 4. Dams. P.C.V. (%).

	-	Cu	Co	Thyroid	Marginal Means
-	53.2	47.2	56.5	56.8	53.4
Fe	39.0	52.2	48.8	50.2	47.6
$CaCO_3$	32.2	36.5	44.8	38.0	37.9
Fe + $CaCO_3$	40.8	38.5	51.2	51.5	45.5
Marginal Means	41.3	43.6	50.3	49.1	46.1

Standard error of difference between { tabular means = ± 5.86; marginal means = ± 2.93

TEXT TABLE 60.

Exp. 4. Dams. M.C.H.C. (%).

	-	Cu	Co	Thyroid	Marginal Means
-	29.18	30.50	28.38	27.38	28.86
Fe	35.85	31.25	31.28	29.60	31.99
$CaCO_3$	27.98	29.62	28.80	29.55	28.99
Fe + $CaCO_3$	25.92	26.22	28.98	24.88	26.50
Marginal Means	29.73	29.40	29.36	27.85	29.08

Standard error of difference between { tabular means = ± 2.50; marginal means = ± 1.25

TEXT TABLE 61.

Exp. 4. Litters. P.C.V. (%).

(a) First litters.

	-	Cu	Co	Thyroid	Marginal Means
-	24.0	19.8	26.8	25.0	23.9
Fe	30.5	34.0	31.5	34.7	32.7
$CaCO_3$	10.0	11.3	15.8	15.0	13.0
Fe + $CaCO_3$	21.3	20.5	17.7	17.3	19.2
Marginal Means	21.5	21.4	23.0	23.0	22.2

(b) Second litters.

	-	Cu	Co	Thyroid	Marginal Means
-	30.8	27.6	35.5	32.8	31.7
Fe	28.7	36.2	41.0	37.8	35.9
$CaCO_3$	13.0	15.0	16.3	15.6	15.0
Fe + $CaCO_3$	19.9	17.9	21.7	31.8	22.8
Marginal Means	23.1	24.2	28.6	29.5	26.4

TEXT TABLE 62.

Exp. 4. Litters. M.C.H.C. (%).

(a) First litters.

	-	Cu	Co	Thyroid	Marginal Means
-	23.4	22.2	25.3	27.3	24.6
Fe	27.8	27.6	29.6	28.4	28.4
$CaCO_3$	20.0	20.8	22.9	23.2	21.7
Fe + $CaCO_3$	22.6	21.0	22.0	25.2	22.7
Marginal Means	23.4	22.9	25.0	26.0	24.3

(b) Second litters.

	-	Cu	Co	Thyroid	Marginal Means
-	29.1	25.7	29.0	31.5	28.8
Fe	30.0	27.5	33.0	28.4	29.7
$CaCO_3$	28.7	24.4	27.4	26.6	26.8
Fe + $CaCO_3$	22.9	24.0	25.0	28.4	25.1
Marginal Means	27.7	25.4	28.6	28.7	27.6

it exceeds the standard error). There is thus important evidence that all three interactions which exceeded the 5% level of significance were in fact real. In interpreting the overall effects, therefore, the influence of these interactions must be considered.

P.C.V. and M.C.H.C. The supplements exerted highly significant effects ($P < 0.001$) on the P.C.V. of the dams (Text Table 59). Most of the variability between the rows was accounted for by the low values in Row 3 (calcium carbonate alone), the calcium carbonate effect being highly significant ($P < 0.001$), while differences between the columns were due almost entirely to the high values given by the diets containing the cobalt and thyroid supplements.

Diet-group means for dams' M.C.H.C., calculated on the statistically adjusted data, are shown in Text Table 60. The differences between the rows were significant ($P < 0.01$), Row 2 (iron alone) being the highest and Row 4 (calcium carbonate plus iron) being the lowest. The differences between the columns were not significant.

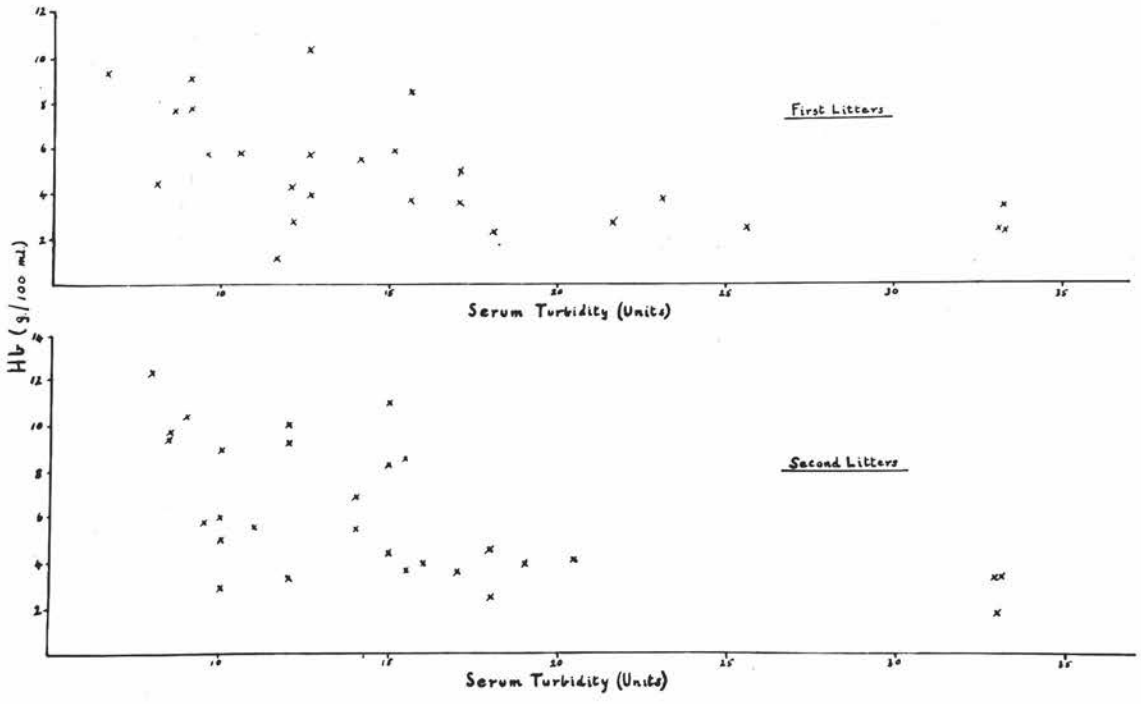
Statistical analysis of the litter data was not attempted because of the large number of missing litters. However, inspection of the unadjusted diet-group means (Text Table 61) suggests very strongly that the calcium carbonate and iron supplements had exerted their expected effects on P.C.V., and also that the cobalt and thyroid supplements had raised the value.

The diet-group means for the M.C.H.C. of litters are shown in Text Table 62. They suggest that calcium carbonate had a lowering effect, and that the cobalt and thyroid supplements may both have increased the value of this attribute.

Serum turbidity. Because of the large number of missing litters, and also because several litters had been so reduced in size that the quantity of serum obtainable from the survivors was insufficient for a satisfactory estimation, it was impossible to compare the readings obtained from individual diet-groups or

FIGURE 31

Exp. 4. Litters. Serum turbidity and blood haemoglobin concentration



TEXT TABLE 63.

Exp. 4. 1st litters. Histological assessment of visible liver fat. Diet-group means

	-	Thyroid	Mean
-	2.0	2.0	2.0
Fe	1.7	1.0	1.4
$CaCO_3$	3.7	4.0	3.9
Fe + $CaCO_3$	2.5	3.0	2.8
Mean	2.5	2.5	2.5

TEXT TABLE 64.

Exp. 5. Allocation of mice to diets

	-				$CaCO_3$			
-	32	12	16	14	23	1	21	20
	6	15	29	13	24	2	10	7
$MnSO_4$	22	28	31	11	25	4	9	5
	18	19	17	3	27	8	26	30

even from their marginal means. However, the data were sufficient to enable an analysis of covariance to be carried out, for first and second litters separately, between turbidity and haemoglobin concentration. The unadjusted data are shown graphically in Fig. 31.

(iii) Histology

Liver fat. As in Experiment 2, the amount of visible fat in the frozen sections was assessed histologically, but, with twelve of the thirty litters missing, statistical analysis of the data was not possible. Nevertheless, inspection of the diet-group means shown in Text Table 63 makes it clear that the thyroid supplement did not abolish fatty change in the liver. It can also be said that the figures accord with the previous finding that dietary calcium carbonate increases liver fat.

Pancreas. None of the specimens of pancreas exhibited any deviation from normal in the structure of their cell-islets. The possibility may therefore be discounted that diabetes mellitus was the cause of the hyperlipaemia and the fatty changes in the liver associated with a high level of dietary calcium carbonate.

4. INTERPRETATION AND DISCUSSION

This experiment, like Experiment 3, was to some extent spoiled by the generally poor rearing performance, which resulted in so many missing observations among the litter data that differences between diet-groups often failed to reach unequivocal levels of significance. The fact that a number of mated pairs failed to breed also diminished the value of the results. Nevertheless, sufficient information was obtained to warrant the drawing of certain conclusions.

The experiment was concerned chiefly with the haematological effects of the experimental treatments, but the effects of the thyroid supplement on reproduction deserve notice. There seems little doubt that this supplement increased the number born, although it also appeared to increase the period from mating to parturition, especially with first pregnancies. The possibility that such a long interval was due to an extended gestation period hardly seems likely, and it must be assumed, therefore, that it was the result of impaired fertility on the part of one or both of the partners.

It seems possible that the level of thyroid supplement may have been too high when the mice were young, especially as up to that time they had been fed on an adequate, non-goitrogenic stock diet; and that the long initial period of non-conception (or possibly conception followed by embryonic death) was due to hyperthyroidism, which, as we have seen, can interfere with fertility. If so, however, it did not interfere with fecundity.

The advantage gained by the thyroid series in 'Number Born' was not translated into a similar advantage in 'Number Weaned' - indeed infant mortality was certainly no lower and if anything slightly higher in this than in any of the other columns. The fact that the surviving weanlings also appeared to be considerably lighter in the thyroid diet-groups suggests that they may have been hyperthyroid (although it could also be that the others were hypothyroid). This does not support the conclusion of Hurst and Turner (1948) that the thyroid hormone is not transmitted through the mammary gland: on the other hand, it is not good evidence against their view, as the infants from the thyroid series may have been lighter at birth, and there is also the possibility that the loss of weight was produced through nibbling their mothers' diet for a few days before weaning.

The thyroid supplement had opposite effects on the body-weights of dams and sires: with the dams, it appeared to minimise gain in weight, while with

the sires it appeared to increase it. The reason for this result is hard to explain. While the feeding of desiccated thyroid would normally be expected to reduce weight, yet there are two reasons for supposing that body-weight could well have been greater in the thyroid series - in the first place, the haemoglobin values were high, thus allowing full tissue development, and, in the second place, owing to the longer 'Mating Periods' the parents in these groups were all older when sacrificed; but these reasons were as true for the dams as for the sires. There thus appears to have been a sex difference in the response to the thyroid supplement; possibly it was connected with the effects of pregnancies in the females.

Because they were based entirely on between-litter comparisons differences between the rows must be interpreted with some reserve in this experiment, and unpredicted effects should not cause undue surprise. This is particularly true of the reproduction attributes, in some of which the calcium carbonate supplement was associated with a superior performance. It has already been pointed out that the infertile matings, whose distribution may well have had nothing whatever to do with diet, could have been the cause of artificial and misleading differences. Nevertheless, although the between-litter random variability was quite large, and so, consequently, was the standard error, the effect of the calcium carbonate supplement was large enough to prove significantly positive on both 'number born' and 'number weaned' with second litters, while a similar trend can also be seen with first litters. Because the results of Experiments 1 and 2, which were based mainly on within-litter comparisons, suggest a quite opposite conclusion, it is worth while investigating this result more closely. On doing so, it is clear that with second litters, on which the effect of calcium carbonate was most pronounced, the largest part of the effect was contributed in column 3 (cobalt); here the reproduction performance of the groups not receiving calcium carbonate was

extremely poor. This would be consistent with the presence of calcium carbonate having moderated a deleterious action exerted by cobalt, but the evidence is of course too meagre to stand on its own.

None of the other effects on reproduction or growth attributes were significant, and in the circumstances it would seem distinctly unwise to try to draw any further conclusions from these data.

On the other hand, with the haematological attributes (particularly Hb) the inter-row relationships proved to be very much as predicted in all four classes of animal - dams, sires, and first and second litters. This fact substantiates the general validity of the haematological data for inter-column comparisons and interactions. In all four classes of animal the haemoglobin concentration (Hb) was, as expected, significantly lowered by the calcium carbonate supplement; the effect was large with all classes except the sires. The iron effect was not so strong; indeed, only with second litters did it reach significance at the 5% level, but with the other three classes it was always positive and fairly large. There can therefore be no reasonable doubt that the ferrous sulphate supplement invariably acted to increase the Hb. The fact that the effect of the ferrous sulphate was apparently less powerful than that of the ferric citrate used in Experiment 2 may or may not be important. The two effects might also be judged by comparing their magnitudes with those of the calcium carbonate effects in each experiment, but such a procedure would be of very doubtful validity because in Experiment 2 the comparisons were mainly within-litter whereas in this experiment they were entirely between-litter. A separate experiment would therefore be needed to settle this point.

Although none of the interactions between iron and calcium carbonate reached significance, it is interesting to note that their general pattern lent support to the hypothesis formulated in the discussion of Experiment 2 -

namely, that calcium carbonate interferes with the action of iron but that this effect will not be apparent if the haemoglobin levels of the control animals are near to the physiological maximum. In the present experiment, first-litter weanlings from the unsupplemented row were distinctly anaemic, and their response to the iron supplement was large (3.98 g./100 ml.); but, although the haemoglobin levels in the row receiving calcium carbonate showed even greater 'room for improvement', iron was much less effective in the presence of calcium carbonate than in its absence. Indeed, if the number of first litters reared had been greater, this difference might well have been significant. With second litters, however, and also with the dams and sires, the haemoglobin levels of the controls were much nearer the physiological maximum, leaving little 'room for improvement'; that this was so is shown by the comparatively small responses given to the inclusion of additional iron in the diets (1.58, 0.32 and 0.49 g./100 ml. with second litters, dams and sires respectively). Consequently, although in all these classes of animal the presence of calcium carbonate had created more 'room for improvement', and the effect of iron had proved to be positive, yet there is no means of telling whether the presence of calcium carbonate had limited the response to the iron. However, the fact that the improvement was never very large suggested that this may well have been the case.

The differences between the columns, taken together, were significant with the Hb of second litters ($\underline{P} < 0.01$) and males ($\underline{P} < 0.05$), but with the dams $\underline{P}$ was about 0.1. Differences with first litters were also large, but because of the many missing observations they did not reach significance. The differences between individual columns must now be considered.

A rather complicated point in the statistical interpretation, for the explanation of which I am indebted to Mr. A. W. Boyne, must be made

at this stage. The levels of probability quoted above refer to probabilities that differences of such magnitude between groups had arisen by chance; but they refer to all the differences taken together, and should not be used to define precisely the significance of a difference between any two particular groups. In other words, although these measures of significance indicate that real differences probably exist, they do not particularise about individual differences. In general, if the greatest of a set of differences be selected, a t test will overestimate its true significance. However, it is a common practice, if not a strictly accurate one, to assign to individual differences a level of significance calculated from t; and there can be no great objection to this practice, especially if its use is restricted to the description of either pre-selected differences or differences which arise in more than one independent set of groupings.

The difference between the copper column and the control column was in no instance large, nor were the interactions between copper and calcium carbonate or iron or both. It is certain, therefore, that the supplement of copper sulphate did not affect, at least to any material extent, the Hb of any class of animal.

The value of Hb in the thyroid column, however, was consistently high; the cobalt column also gave high values with the dams and with both first and second litters, and in these classes the thyroid and cobalt series between them account for much the greater part of the total variability. With the dams, for instance, the difference between the combined means of the cobalt and thyroid columns on the one hand, and the combined means of the copper and control columns on the other, amounts to 1.42 g./100 ml., which difference may be taken as significant at the 5% level (the standard error, with 34 degrees of freedom is ± 0.57). This procedure yields a similar result with second litters, where the difference is 1.89 g./100 ml. ($P < 0.01$), while with first litters the difference (0.87 g./100 ml.) is in the same direction though not large enough to be considered significant. With the males, a similar comparison cannot be made, as the overall mean of the cobalt column is not greater than that of the control column (an explanation of this result will be promoted later when the interactions are considered). It is true that three combinations

of effects were possible but only one has been selected, and thus the levels of significance quoted may be exaggerated for the reasons explained above; but, because the trend was evident in three of the four classes, it is reasonable to assume that it was a real one.

It may therefore be said that the cobalt sulphate and desiccated thyroid supplements increased the Hb of dams and litters, but that with the males differences were too small to warrant any firm conclusions being drawn.

In presenting the results, however, it was shown that three interactions had reached at least the 5% level of significance, and also that their influence must be considered in interpreting the results. One of these was the interaction on sires' Hb between cobalt sulphate and calcium carbonate, which was significant at the 5% level. Whereas with the other classes of animal cobalt appeared to increase Hb, with the males there was no overall increase - if anything, a slight decrease; but the interaction shows that cobalt reduced the value unless calcium carbonate was also present, the separate effects being -1.11 and +0.56 respectively, each having a standard error of ± 0.53. Obviously, then, cobalt had a toxic effect on the sires' Hb ($P < 0.05$), while calcium carbonate acted as an antidote; and this would be consistent with the suggestion made on p.139 that a high level of calcium carbonate in the diet might interfere with the availability of dietary cobalt. It would also seem that the level of cobalt supplement must have been toxic to the sires but less so to the dams, for with these animals the overall effect of the cobalt supplement was positive (as it was in Experiment 3). But here the same interaction is to be found, and at a high level of significance ($P < 0.01$); the separate effects in the absence and presence of calcium carbonate are -0.75 and +4.17 respectively, each with a standard error of ± 1.2. The action of cobalt alone in this case is not significant, but cobalt

in the presence of calcium carbonate had a highly significant positive effect ($P < 0.001$). Together with the interactions observed with the reproduction data, this result gives further strong support to the theory that dietary calcium carbonate can interfere with the availability of cobalt. This interaction was not significant with either of the litters, but in each case the effect of cobalt was slightly more positive in the absence than in the presence of calcium carbonate (the respective effects being, first litters +1.33 and +0.33, both $\pm$0.89; second litters +1.33 and +1.13, both $\pm$ 0.79). These effects also are not significant, but they do nothing to refute the hypothesis and if anything tend to support it.

The other significant interaction was that between thyroid and calcium carbonate on the Hb of the dams. Thyroid had no effect in the absence of calcium carbonate, but in its presence the Hb was significantly raised ($P < 0.01$); the separate effects were -0.52 and +3.26 respectively, the standard error of both being $\pm$1.12. This result could mean that the thyroid supplement had the property of raising the Hb, but that the presence of calcium carbonate was also necessary for its utilisation; equally, however, it could mean that thyroid was ineffective when the Hb was already high, but could partially maintain the value when this became subjected to the lowering influence of calcium carbonate. A similar trend can be observed in the data from the males, the separate effects of thyroid on Hb being +0.11 in the absence and +0.91 in the presence of calcium carbonate, the standard error being $\pm$0.53 in each case. But this trend was not seen with the first litters, which differed from the adults in that the values in the control groups were far from maximal; indeed the effect was slightly more positive - though not significantly so - in the absence of calcium carbonate (+1.55 $\pm$ 0.87) than in its presence (+0.70 $\pm$ 0.87). With second litters, on the other

hand, the effect of thyroid in the absence of calcium carbonate was +1.38 ± 1.07, that in its presence being +3.00 ± 1.07; the latter effect was itself highly significant ($P < 0.01$), though it was not significantly different from the former. The pattern thus resembled that seen with the dams and sires rather than with first litters, and in this connection it is interesting to remember that with second litters the general performance was much improved, and the values of the control groups were much higher than with first litters. The more likely explanation of the interaction would thus seem to be that the thyroid supplement was effective only when the value in the control group was sub-maximal; but, of course, the experiment was too small to provide conclusive evidence on this point. However, because the thyroid supplement succeeded in raising the Hb in some cases, it follows that, directly or indirectly, it must have enabled fuller utilisation to be made of whatever iron was available.

That the general pattern of the Hb data was reflected in that for P.C.V. is not surprising, but it is interesting to note that the increased Hb of the dams in the cobalt and thyroid groups was not associated with a comparable increase in M.C.H.C. This may have been because the M.C.H.C. was already practically maximal in the majority of these animals. With the litters, on the other hand, and especially with first litters, neither the Hb nor the M.C.H.C. were maximal, and both attributes appear to have been raised by both cobalt and thyroid.

Although the results of the serum turbidity estimations were so few and so variable that only very limited information could be obtained from them, nevertheless with first litters a negative correlation was established between serum turbidity and blood Hb, thereby confirming the trend seen in all previous experiments.

The results of the histological observations call for little comment. Clearly neither hypothyroidism nor diabetes mellitus was responsible for the

disturbances in fat metabolism associated with high levels of dietary calcium carbonate.

5. SUMMARY

1. The object of the experiment was to observe the effects on the haemoglobin concentration and other haematological attributes of breeding mice and their young of adding supplements of copper sulphate, cobalt sulphate and desiccated thyroid to a slightly modified Sherman B diet; and whether these effects were varied by the presence of ferrous sulphate, calcium carbonate, or both. Supplementary observations were also to be made on reproduction performance and weight gain.

2. The reproduction performance, particularly rearing performance, was very poor in all groups, and in consequence the accuracy of all litter data was much diminished.

3. Thyroid increased the number born, but it also increased the period between mating and parturition; it did not affect survival rate. The level of supplementation employed may have been so high as to cause hyperthyroidism.

4. Thyroid reduced the weight of the young at weaning. It appeared to minimise weight gain in the dams but to increase it in the sires; the reason for this is not clear.

5. Calcium carbonate reduced the blood haemoglobin concentration in all classes of animal, while ferrous sulphate increased it with second litters and probably increased it with the other classes. It is not possible to say from this experiment whether the ferrous sulphate supplement was as effective in this respect as was the ferric citrate used in Experiment 2.

The general pattern of the Fe x $CaCO_3$ interaction lent support to the hypothesis that calcium carbonate interferes with the utilisation of iron.

6. Copper sulphate did not affect to any material extent the blood haemoglobin concentration of any class of animal.

7. In general cobalt sulphate and thyroid both increased blood haemoglobin concentration in all classes; but the effect of cobalt sulphate was hindered by the additional presence of calcium carbonate, and that of thyroid was evident only when the control value was low - as it always was with the litters, and also was with the parents when the diet contained calcium carbonate. With the sires, the cobalt supplement was toxic unless its effects were moderated by the presence of calcium carbonate.

8. Data for packed cell volume in general followed the pattern of the data for haemoglobin concentration; but even where this was raised by the cobalt and thyroid supplements, the mean cell haemoglobin concentration was not similarly increased unless the value of the control group was low.

9. With first litters, a negative correlation was established between serum turbidity and blood haemoglobin concentration.

10. The quantity of visible fat in the liver was not affected by the thyroid supplement.

11. The cell-islets of the pancreas in both dams and weanlings were not affected by the calcium carbonate supplement.

EXPERIMENT 5.

This experiment was carried out, following the failure of Experiment 3, to

determine whether or not a supplement of a manganese salt in the diet would prevent the impairment in reproduction and rearing performance associated with high levels of calcium carbonate in the diet. The reasons prompting a study of this element have already been explained.

1. Construction of Diets

As in the preceding experiment, the basal diet employed was Diet 2, with Diet 69 as the high calcium carbonate control. Two experimental diets, each containing about 5 p.p.m. of manganese, were prepared by adding 2.13 mg. of manganous sulphate ($MnSO_4.4H_2O$) to each 100 g. of Diet 2 and to each 102 g. of Diet 69.

2. Procedure and Methods

Thirty-two female mice were employed, but, as insufficient sets of littermates were available, the mice were distributed equally among the four diets at random. To accomplish this, the mice (at seven weeks of age) were weighed and numbered in descending order of weight, and allotted to the four diets by drawing lots (Text Table 64). The mice were fairly uniform in weight, the mean weights of the animals in each diet-group being as follows: Diet 2, 21.9 g.; Diet 2 + Mn, 21.8 g.; Diet 69, 22.8 g.; and Diet 69 + Mn, 22.1 g.

The animals were introduced to the experimental diets immediately, i.e. at seven weeks of age; one week later they were mated, two sets of four littermate males being used for this purpose. One male from each set was allotted to each diet-group, and was mated with four of the females in large breeding cages. When seen to be pregnant, the females were transferred to smaller separate cages.

A careful record was kept of all reproduction data, and the young were weighed on weaning at 21 days old. Although there was no evidence that

TEXT TABLE 65.

Exp. 5. Summary of results

Mice nos. \ Diets	No. born				No. weaned			
	2	2 + Mn	69	69 + Mn	2	2 + Mn	69	69 + Mn
32/22/23/25	8	2	8	2	2	0	8	0
12/28/ 1/ 4	7	9	11	8	3	8	6	7
16/31/21/ 9	10	9	11	8	8	7	9	1
14/11/20/ 5	7	5	9	11	6	4	0	0
6/18/24/27	8	8	7	9	8	7	0	8
15/19/ 2/ 8	8	8	11	...	8	7	0	...
29/17/10/26	6	4	10	8	6	4	7	7
13/ 3/ 7/30	8	8	10	...	7	7	0	...
Total	62	53	77	46	48	44	30	23
Mean	7.8	6.6	9.6	7.7	6.0	5.5	3.8	3.8

Mice nos. \ Diets	Total wt. weaned (g.)				Hb (g./100 ml.); litter means			
	2	2 + Mn	69	69 + Mn	2	2 + Mn	69	69 + Mn
32/22/23/25	14.4	0	37.1	0	5.92	-	2.22	-
12/28/ 1/ 4	21.4	65.4	42.5	40.6	2.66	6.51	3.55	2.22
16/31/21/ 9	45.6	32.5	47.0	3.5	5.33	6.07	2.66	2.22
14/11/20/ 5	41.0	44.2	0	0	3.26	5.03	-	-
6/ 8/24/27	58.4	54.6	0	33.0	9.92	4.74	-	2.22
15/19/ 2/ 8	44.8	65.7	0	...	6.51	6.96	-	...
29/17/10/26	60.0	43.6	38.8	38.8	7.40	4.29	2.37	2.52
13/ 3/ 7/30	50.0	61.4	0	...	3.85	4.88	-	...
Total	335.6	367.4	165.4	115.9	44.85	38.48	10.80	9.18
Mean	42.0	45.9	20.7	19.3	5.61	5.50	2.70	2.30

Attributes \ Diets	2	2 + Mn	69	69 + Mn
Survival rate (%)	77	83	40	50
Mean wt. per weanling (g.)	7.0	8.4	5.5	5.0

manganese can affect blood haemoglobin concentration, this attribute was nevertheless measured (by Haldane's method) on representatives of each weaning litter.

3. Results

A summary of the results is presented in Text Table 65.

Only one of the thirty-two does failed to become pregnant, but one of the remaining animals aborted. Both were on Diet 69 + Mn. Seven dams failed to rear their litters, six of them being in the high calcium carbonate diet-groups.

It can be seen from the Table that the calcium carbonate effect was, as expected, markedly negative with every attribute except 'Number born'. The manganese effect, on the other hand, was very small, and not consistent; there can be no doubt that this supplement did not materially affect rearing performance or haemoglobin concentration.

4. Conclusions

No evidence was obtained that a supplement of manganese can abolish the effects of a high level of dietary calcium carbonate. It follows that the improvement in rearing performance observed in Experiment 2 when the diet was supplemented with a preparation of ferric citrate could not have been caused by contaminating manganese.

CONCLUSIONS

The experimental work described in this section has given some support to the hypothesis that calcium carbonate interferes with the utilisation of iron, and has shown that while a supplement of Analar ferrous sulphate in the Sherman B diet raises the haemoglobin concentration of mated pairs of mice and their young, this property is also possessed by cobalt sulphate. It has also been

shown that dietary calcium carbonate interferes with the effects of cobalt, and so it must be concluded that some of the effects of the ferric citrate used in Experiment 2 could possibly have been due to contamination with cobalt. On the other hand, contamination with copper or manganese could clearly have played no part, as experiments have shown that supplementing the diet with copper or manganese has no important effects on the haemoglobin level or the reproduction performance.

It has also been shown that desiccated thyroid can raise the low haemoglobin level associated with the addition of a supplement of calcium carbonate to the diet. From this it can be surmised that dietary calcium carbonate may induce hypothyroidism, and that its action in causing anaemia may be partly accounted for by this property. In these experiments, desiccated thyroid also increased the numerical size of the litters at birth, but, on the other hand, did not improve rearing performance, and indeed it had an adverse effect on the growth of the young animals; moreover, it did not influence the amount of visible fat in their livers. Thus, the effects of calcium carbonate on these attributes do not appear to result from hypothyroidism.

The work has also shown that the fatty livers and hyperlipaemia associated with high levels of dietary calcium carbonate are not caused by diabetes mellitus.

E. DIETARY CALCIUM CARBONATE AND PYRIDOXIN

Among the causes of hypochromic anaemia is a deficiency of pyridoxin (Vitamin B_6); and in considering this vitamin it was stated that it could not be excluded as a possible cause of the anaemia seen in the experimental mice from groups fed high levels of calcium carbonate.

> The erythropoetic action of this vitamin was first reported by Fouts, Helmer, Lepkovsky and Jukes (1938) who fed puppies on a purified casein diet and supplied them with vitamins other than pyridoxin. The animals failed to grow properly, all developed severe microcytic hypochromic anaemia, and some developed convulsions. By feeding this same diet, Fouts, Helmer and Lepkovsky (1939, 1940) subsequently produced a microcytic hypochromic anaemia in adult dogs also. In both puppies and adults, the anaemia was curable by the addition to the diet of crystalline vitamin B_6.
>
> Similar findings in dogs were reported by McKibbin, Madden, Black and Elvehjem (1939-40). Chick, Macrae, Martin and Martin (1938) noted microcytic anaemia and epileptic fits in pigs fed on a synthetic diet, and were able to cure these abnormalities by administering the eluate fraction prepared by Edgar and Macrae (1937), which contained vitamin B_6.
>
> The action of pyridoxin has not yet been properly elucidated, but Cartwright and Wintrobe (1948) have suggested that in pyridoxin deficiency the fundamental disturbance in erythropoiesis is a failure to synthesise protoporphyrin; they found a significant lowering of free protoporphyrin in the red cells of pyridoxin-deficient pigs. In reviewing past work, these authors described the morphology of the blood in the anaemia of pyridoxin deficiency: the anaemia "is characterised by microcytosis, slight hypochromia, and an increase in polychromatophilia, reticulocytes and nucleated red cells in the blood". They also describe the bone marrow as exhibiting normoblastic hyperplasia.

In view of these facts, it seemed possible that a deficiency of pyridoxin might have played a part in the causation of the anaemia seen in my experimental mice. In some ways, however, it seemed unlikely that this could have been the case. The Sherman B diet contains a large proportion of whole wheat, which is a good source of the vitamin B complex, including pyridoxin (Richards, 1949b); further, in my animals there had been no evidence of the convulsive fits which are usually said to accompany pyridoxin deprivation.

On the other hand, Richards (1945, 1946, 1949a) has repeatedly observed, in rats, that the effects of pyridoxin deficiency (caused by feeding an excess of aneurin) were aggravated when the diet was supplemented with various levels of calcium carbonate, or other salts of calcium. Like Stoerk and Zucker (1944) and Stoerk (1946), she also observed (Richards, 1949a) that pyridoxin has a specific effect on thymus development, and, indeed, in comparing the occurrence of fits and the atrophy of the thymus in pyridoxin deprivation, she stated that "atrophy of the thymus gland appears to be the surer indication of deficiency, since it may be found even in well-grown weanlings which have shown no outward sign of the deficiency." Another feature of pyridoxin deficiency described by Richards (1949b), is a very high death-rate among infants born of deficient dams.

Now, my experimental mice had been fed on diets high in calcium carbonate, which, as we have just seen, may have the property of inducing a deficiency of pyridoxin. Not only had they developed an anaemia very similar in type to that regularly found in pyridoxin deficiency, but they had failed to rear many of their young and, moreover, the survivors had exhibited thymus atrophy. I therefore considered it important to determine whether a supplement of pyridoxin such as had cured Richards' rats would have any effect on the anaemia in my mice, and accordingly the following experiment (Experiment 6) was carried out.

EXPERIMENT 6.

1. Construction of Diets

The control diets employed were Diets 79 and 81, as used in Experiment 2 and the two experimental diets were formed by the addition of a supplement of pyridoxin to each control diet. In order to ensure even mixing throughout the diet, I decided to dissolve the pyridoxin in the distilled water used in preparing

the dough.

Richards (1949b) had found 40 μg. of pyridoxin per animal per day to be sufficient to overcome a deficiency in rats, and it might therefore be expected that, for mice, a daily dose of 20 μg. per animal would have been ample. In view of a possible species difference, however, and also because the vitamin was to be given by mouth instead of by injection, I selected a level equal to five times this amount.

On the assumption that the average daily food intake was 10 g. per mouse, this dose could be administered by incorporating 1 mg. of pyridoxin into each 100 g. of the ration. Accordingly, 30 mg. of pyridoxin were dissolved in each litre of the distilled water used in preparing the dough.

2. Procedure and Methods

Thirty-two female mice, comprising eight sets of four littermates, were bred in the usual way. When seven weeks of age, one member of each litter was assigned at random to each of the four diets. One week later they were mated monogamously with males of similar age. The males also consisted of eight sets of four littermates; the individual members of each litter of males were mated with the members of one litter of females, within-litter distribution again being random. There were thus eight replicates of a simple 2^2 design, with all comparisons within-litter.

Records were kept of the reproduction data and of the mating weights and final weights of the parents. The sires were allowed to remain with the dams until the latter were pregnant for the second time. Blood haemoglobin concentration was estimated on the sires when they were removed from the mating cages, and on the dams on the day their second litter weaned (or, if the litter did not survive until weaning age, on the day that the last of the infants succumbed). Haemoglobin estimations were made on the young mice at 21 days of

Exp. 6. Diet-group means and effects.

Attribute	Class	Means				Effects	
		-	$CaCO_3$	Pyridoxin	Pyridoxin + $CaCO_3$	$CaCO_3$	Pyridoxin
No. of litters born	1st litters	8	8	8	8	-	-
	2nd litters	8	8	8	8	-	-
No. of litters weaned	1st litters	7	5	6	5	- 1.5	- 0.5
	2nd litters	7	5	6	5	-1.5	-0.5
No. of young born	1st litters	7.9	7.3	6.9	7.6	+ 0.1	- 0.4
	2nd litters	6.5	6.5	7.1	6.1	-0.3	+ 0.1
	Mean	7.2	6.9	7.0	6.8	- 0.1	- 0.2
No. of young weaned	1st litters	4.3	4.0	5.1	2.1	- 1.7	- 0.6
	2nd litters	4.9	3.6	5.3	2.0	- 2.3	- 0.6
	Mean	4.6	3.8	5.2	2.1	- 2.0	- 0.6
Survival rate (%)	1st litters	54	55	75	28	-23	- 3
	2nd litters	87	74	77	37	-27	-24
	Mean	71	65	76	33	-25	-14
Wt. weaned/litter born (g.)	1st litters	29.5	23.1	32.1	10.6	-14.0	- 5.0
	2nd litters	46.4	26.2	46.5	12.0	-27.4	- 7.2
	Mean	38.0	24.7	39.3	11.3	-20.7	- 6.1
Mean wt./weanling (g.)	1st litters	7.2	6.5	6.3	5.1	- 1.0	- 1.2
	2nd litters	9.5	7.9	9.0	6.5	- 2.1	-1.0
	Mean	8.4	7.2	7.7	5.8	- 1.6	-1.1
Hb (g./100 ml.)	1st litters	3.98	2.81	3.55	2.46	- 1.14	- 0.40
	2nd litters	4.50	2.65	4.19	2.47	- 1.81	- 0.25
	Mean	4.24	2.73	3.87	2.47	- 1.47	- 0.33
Mating wt. (g.)	Dams	24.4	24.4	24.2	24.3	+ 0.1	- 0.2
	Sires	26.1	26.5	26.0	25.7	+ 0.1	- 0.5
Final wt. (g.)	Dams	34.7	31.9	35.2	32.1	- 3.0	+ 0.4
	Sires	34.8	33.3	34.2	34.0	- 0.9	+ 0.1
Hb (g./100 ml.)	Dams	11.74	10.45	11.16	10.77	- 0.84	- 0.13
	Sires	13.60	13.08	13.10	13.39	- 0.12	- 0.10
Thymus wt. (mg./100 g. body wt.)	1st litters	341	222	331	236	-107	+ 2
	2nd litters	318	167	294	159	-143	-16

age, at which time their thymus glands were also examined and weighed.

In all cases, haemoglobin concentration was estimated colorimetrically (see Technical Appendix, p.251).

3. Results and Discussion

A summary of the results is presented in Text Table 66.

All does bore two litters, but nine first and nine second litters failed to survive. The distribution of these 'failures' among diet-groups happened to be similar with both first and second litters, but the same dams were not involved on both occasions; four dams - one on each diet - failed to rear both their litters, from which it can be gathered that five others lost their first litters and a further five lost their second litters. Thus, of the thirty-two does, only eighteen succeeded in rearing two litters or part-litters.

The effects of the calcium carbonate supplement proved to be very much as could have been predicted, being almost invariably negative and being most marked on the thymus weights of litters, the rearing performance, and the haemoglobin concentration of litters and dams. If statistical analysis of the data had been necessary, it would have been rendered difficult by the rather poor reproduction performance. However, the data show quite clearly, from inspection alone, that with no attribute did the pyridoxin supplement prove helpful; indeed, in practically every instance its effects were slightly negative in direction, and they even suggest that possibly pyridoxin had had a toxic effect. However, on only two occasions were the effects at all sizable - on the survival rate of second litters and on the haemoglobin concentration of first litters. Probably, therefore, the negative effects were due to chance - the fact that the attributes are not independent will largely account for the consistency in direction.

In any case, however, even if it were true that pyridoxin had been toxic

in the doses employed, it is clear that calcium carbonate did nothing to alleviate such an effect; for, as can be seen from the Table, in general the negative effects of pyridoxin were stronger in the presence than in the absence of calcium carbonate. From this it could be inferred that calcium carbonate did not hinder any action of the vitamin.

Thus, if the dose of pyridoxin used in this experiment had indeed been so large as to be toxic, then it can be argued that there would be no basis for supposing that calcium carbonate could have induced a pyridoxin deficiency in Experiments 1 and 2. If, on the other hand (and as seems more likely), it had not been toxic, then it has been shown that the addition of pyridoxin to the diet did nothing to abolish the effects of calcium carbonate. It may therefore be concluded from this experiment that, whether or not pyridoxin had a toxic action in the dose here employed, the deleterious effects of calcium carbonate on reproduction, thymus weight and haemoglobin concentration were in no way associated with an induced deficiency of pyridoxin.

F. DIETARY CALCIUM CARBONATE, PHYTIC ACID AND INOSITOL; AND IODINE

The polyhydric alcohol inositol (or 'inosite') is now recognised as a vitamin and is usually considered as part of the Vitamin B complex. Under normal dietary conditions (especially when the ration contains a cereal) deficiency of this substance is unlikely to occur, as it is included in the molecule of phytic acid.

Phytic Acid

The isolation of phytic acid was first achieved by Posternak (1903) who obtained "un acide phospho-organique" from the seeds of various cereals, legumes, bulbs and rhizomes. Patten and Hart (1904) isolated a similar acid ('anhydro-oxymethylene-diphosphoric acid') from wheat bran, and stated that 85% of the phosphorus in wheat bran was in this form. The acid has come to be known as phytic acid, (or inositol hexaphosphoric acid) and its salts as phytates. Anderson (1915) gave its composition as $C_6H_{18}O_{24}P_6$. One of the most important compounds of phytic acid found in nature is its double salt of calcium and magnesium, known as phytin.

Phytin is extremely insoluble, is not hydrolysed by dilute acid at 37° C. (Plimmer, 1913b), but may be hydrolysed under the influence of an enzyme, phytase. Mellanby (1929) also found that phytates were resistant to the action of diastase, and - at least partially - to that of trypsin. Suzuki, Yoshinuira and Takaiski (1906) first reported the existence of a phytase in wheat bran, a finding confirmed by Plimmer (1913a). The existence of such an enzyme was also suspected by Hart, McCollum and Fuller (1908-9), who found that neither pepsin nor trypsin had the power to hydrolyse phytin, but yet observed in the pig that when dietary phosphorus was entirely organic, with 70% of it consisting of phytin, the form of excreted phosphorus was almost wholly inorganic. Phytases have also been recovered from malt (Adler, 1916), rice bran (Horiuchi, 1931) and calf liver (McCollum and Hart, 1908).

It is now established that phytase is present in practically all cereals. McCance and Widdowson (1944b) stated that rye contains most, followed by wheat, barley and oats in that order. However, Møllgaard, Lorenzen, Hansen and Christensen (1946) have shown that these phytases are not all equally effective, as while incubation at 40° C. for two hours results in complete hydrolysis of all the phytin in wheat, the same treatment affects only 8% of the phytin in oats, and an even smaller proportion of that in maize.

Although Mendel and Underhill (1906-7) claimed that the sodium salt of phytic acid was readily absorbed, there is no phytase in the blood of mammals (Rapoport, Leva and Guest, 1941). Many attempts to isolate a phytase from the intestinal wall of animals have been unsuccessful (Plimmer (1913a) using the dog, cat, sheep, ox and rabbit; Lowe and

Steenbock (1936a, b) using the rat; Patwardhan (1937) using the rabbit and guinea-pig). However, Patwardhan (1937) claimed to have obtained a phytase from the intestine of the rat, as did Spitzer and Phillips (1945a). This phytase activity rendered the phosphorus of soybean meal available to the rat (Spitzer and Phillips, 1945b) although the meal itself contained no phytase. The latter authors also obtained a phytase in the rumen and intestinal wall of the bovine, and Reid, Franklin and Hallsworth (1947) also found that phytin was hydrolysed in the rumen, although they did not report the isolation of a phytase. Spitzer and Phillips (1945a) found phytase activity in the intestinal contents, but not the intestinal wall of the pig, and also in the contents of the small intestine of the chicken (Spitzer and Phillips, 1945c).

These workers also pointed out that a further source of phytase activity might be the intestinal flora, a suggestion previously put forward by Lowe and Steenbock (1936b). On the other hand, Pederson (1941) suggested that there may be some relationship between the availability of phytin-phosphorus and the phytase activity of the ration; and Singsen and Mitchell (1944) found that the phytin-phosphorus of soybean meal became relatively more available to the chicken when field-cured alfalfa-leaf meal was also fed, and they believed its action was due to the fact that it was a source of phytase; dehydrated alfalfa-meal was ineffective, and they presumed that this was because phytase had been destroyed during processing. (McCance and Widdowson (1944b) have confirmed that, when wet, it is instantly destroyed at 90° C.) But Boutwell, Geyer, Halverson and Hart (1946) pointed out that vitamin D may have been the active constituent of the fresh meal, since this vitamin has ~~been~~ a favourable influence on phytin-phosphorus utilisation (see below), and they found that this was unaffected by the presence or absence of phytase in the diet. Singsen and Mitchell (1945) later agreed that vitamin D had this action, and found in addition that irradiated animal sterols were even more effective.

Although a certain amount of inorganic phosphate is normally released from ingested phytate (Lowe and Steenbock, 1936b; Hoff-Jørgensen, 1946; Hoff-Jørgensen, Andersen, Begtrup and Nielsen, 1946; Hoff-Jørgensen, Andersen and Nielsen, 1946), the availability of the phosphorus in phytates is nevertheless low (Bruce and Callow, 1934; McCance and Widdowson, 1935; Lowe and Steenbock, 1936a; Krieger, Bunkfeldt and Steenbock, 1940a). However, Mellanby (1950) has stated that vitamin D controls the conversion of phytate to inorganic phosphate, probably by promoting its hydrolysis under the influence of a phytase; and certainly there is ample evidence that the availability of phytin-phosphorus is increased in the presence of vitamin D (Lowe, Steenbock and Krieger, 1939; Krieger, Buckfeldt and Steenbock, 1940a; Krieger and Steenbock, 1940; Krieger, Buckfeldt, Thompson and Steenbock, 1941; Heuser, Norris, McGinnis and Scott, 1943; McGinnis, Norris and Heuser, 1944; Singsen and Mitchell, 1945). On the other hand, excessive amounts of calcium in the diet inhibit the hydrolysis of phytin (Lowe and Steenbock, 1936b; Snook, 1938; Krieger and Steenbock, 1940; Cruikshank, Duckworth, Kosterlitz and Warnock, 1945-6), although when the level of dietary calcium is low, the calcium in calcium phytate is as readily available as that in calcium carbonat (Krieger, Buckfeldt and Steenbock, 1940b). This fact points to one of the most important properties of phytic acid, namely its ability to precipitate

calcium and also certain other metals, notably iron. Yang (1940) has worked out the relative proportions of calcium and phytate required to cause precipitation, and has shown that if the quantity of calcium present in a mixture is less than half the equivalent of sodium phytate, no precipitation takes place; when the quantity of calcium is between half and one equivalent the solution becomes turbid; while a precipitate forms, leaving the supernatant liquid clear, when calcium is present in excess.

The great affinity of phytic acid for calcium is the basis of the belief that the rachitogenic properties of cereals, especially oatmeal (Mellanby, 1925; Burton, 1929-30), depend largely if not entirely on their phytic acid content (Harrison and Mellanby, 1939; Mellanby, 1949). (For a review of the earlier work, see Anonymous, 1941; also Mellanby, 1950). Phytates given by mouth, whether alone or in bread, have been shown repeatedly to induce a negative calcium balance (McCance and Widdowson, 1942-3a, b; Krebs and Mellanby, 1943; Hoff-Jorgensen, 1946; Hoff-Jorgensen et al., 1946, 1946). Even if phytates fail to precipitate all the dietary calcium, they may render much of it unionised, thus impeding its absorption (Harrison and Mellanby, 1940), and it has long been known that the addition of a calcium salt to a cereal diet produces beneficial effects on calcium absorption (Mellanby, 1925). It was for this reason that calcium carbonate was added to the high-extraction flour used in Great Britain during the war of 1939-45 (M.R.C. Memoranda, 1940, 1941; McCance and Widdowson, 1942-3a). The necessity for this practice has been questioned, however, as Pringle and Moran (1942) have shown that the phytic acid in wheatmeal dough can be almost completely destroyed by slight reduction of the pH of the crumb, while much also disappears if the dough is merely allowed to stand for some time at ordinary temperatures. Mellanby (1944) himself realised that the milling and baking processes destroyed much of the phytic acid in wheat. Furthermore, Walker, Fox and Irving (1948) have claimed that the body can adjust itself to high intakes of phytate, and that "consumption of such a diet over long periods has no deleterious effect upon calcium metabolism".

Phytic acid and phytates in the diet also interfere with the absorption of iron. Ferric phytate is highly insoluble, and Nakamura and Mitchell (1943), who reported that iron fed in this form was much less available to rats than was iron in other forms, suggested that this fact may explain why the iron in wheat is apparently poorly utilised. Widdowson and McCance (1942) had previously come to this conclusion on finding that wheatmeal flour, despite its containing larger amounts of iron, was less satisfactory as a source of iron for human subjects, and they considered the most likely reason for this to be the high phytate and/or phosphate content of the unextracted flour. When they incorporated sodium phytate into white bread, and fed it to human subjects, they found (McCance, Edgecombe and Widdowson, 1943) that this procedure prevented the rise in serum iron normally observed following the consumption of large doses of soluble iron salts. Again, when sodium phytate was added to the diet of anaemic rats, Harrison (1942) observed a diminution (although only a small one) in the haemoglobin response to an iron and copper supplement. Sharpe, Harris, Peacock and Cooke (1948) and Sharpe, Peacock, Cooke and Harris (1950) also found, this time in schoolboys, that the addition of sodium phytate to a test meal markedly reduced the absorption of radio-active iron. However, they also observed that this reduction was five times as great as that caused by oatmeal containing the same quantity of phytate,

and they further pointed out that there is evidence of an inverse correlation between iron absorption and the solid content of the meal. In their experiments, the consumption of milk also hindered iron absorption to about the same extent as did oatmeal. Walker, Fox and Irving (1948), on the other hand, could find no evidence that high extraction bread affected iron retention (although this must in any case have been very small, as his three human subjects were all men), and Cullumbine, Basnayake, Lemottee and Wickramanayake (1949-50) were also unable to note any difference in iron retention depending on whether polished or unpolished rice was fed; but again their subjects were men, whose normal iron requirements are very small indeed.

The effect on iron availability of adding small amounts of calcium to diets high in phytate has been reported by Widdowson and McCance (1942); they found that it certainly did not interfere with iron absorption, and if anything appeared to facilitate it, and Cruikshank, Duckworth, Kosterlitz and Warnock (1945-6) later came to the same conclusion.

From the above review we can conclude that phytic acid (or inositol hexaphosphoric acid) is found in cereals, and requires an enzyme, phytase, to hydrolyse it into inositol and inorganic phosphate. Phytase is found in the cereals themselves, and may also be secreted by the intestine of animals or be synthesised by their intestinal flora. The presence of vitamin D assists the breakdown of phytate, but the presence of calcium hinders it. Phytic acid has a great affinity for calcium, and can precipitate or prevent the ionisation of this metal, so inducing a calcium deficiency; on the other hand, if calcium is present in excess the impaired hydrolysis of phytate may result in the development of a deficiency of inorganic phosphate. Phytic acid also has a great affinity for iron, and so may render dietary iron unavailable; the addition of calcium to diets high in phytate can partially offset this effect.

About two-thirds of the Sherman B diet consists of whole wheat, which contains about 0.38% of total phosphorus. Patten and Hart (1904) stated that over 85% of the phosphorus in wheat bran is organic, and Møllgaard et al. (1946) obtained an almost identical figure - 86% - for the proportion of phytic acid phosphorus to total phosphorus in both whole wheat and wheat bran; the estimate of McCance and Widdowson (1935) was somewhat lower. Thus the Sherman B diet

must be rich in phytic acid.

It might therefore seem that the phytic acid in the basal diet used in Experiments 1 and 2 could have interfered with iron availability; but in that case the addition of calcium carbonate should have released the iron and rendered it more available. In fact, however, we have seen that almost certainly it had the reverse effect.

On the other hand, there is no reason why the calcium carbonate should not have precipitated much of the phytate as the calcium salt. It is true that some of the phytate may have been hydrolysed under the influence of the phytase in the wheat when the ration was kneaded into a water dough prior to feeding, but as the calcium carbonate was also mixed in the ration it is unlikely that very much could have escaped precipitation.

In that case, the question of whether a deficiency of the products of phytate hydrolysis could have arisen requires examination. These products are inositol and inorganic phosphate. It has already been shown in this series of studies that a supplement of inorganic phosphate did not abolish the deleterious effects of the dietary calcium carbonate, so clearly a deficiency of inorganic phosphate was not the main cause of the disorder.

However, the possibility that a deficiency of inositol was concerned must now be studied in detail.

Inositol

Inositol ('inosite' or 'cyclohexanehexol') may be regarded as a polyhydric alcohol. Its nutritional significance for animals has been realised only quite recently (Woolley, 1944; Norris and Hauser, 1945); much earlier, Anderson (1916) and Anderson and Bosworth (1916) studied the effects of administering inositol to dogs and man, but found that it had no appreciable effect on general metabolism.

The first reference to the biological importance of this substance was made by Eastcott (1928) who recognised that "Wildiers' Bios (Fraction I of Lucas)", a factor known to be necessary for the normal reproduction of yeast, was in fact meso-inositol. The importance of inositol in animal

nutrition was first recognised when it was discovered (Woolley, 1940a) that young mice fed on a purified diet ceased to grow and became completely hairless over the trunk. Norris and Hauschildt (1940) described a similar syndrome. The condition could be cured by a factor in liver extract, later recognised as inositol (Woolley, 1940b, 1941a); compounds containing inositol, such as phytin, were also effective for the mouse (which could apparently hydrolyse them), but not for yeast, but of the inositol isomers only meso-inositol (i-inositol) was active (Woolley, 1941b).

The distribution of the alopecia of inositol-deficiency in mice appears to be characteristic (Martin, Thompson and de Carvajal-Forero, 1941), but the incidence of the symptom is not usually above 50% (Woolley, 1941c) and may not even appear at all, although death invariably follows precipitous loss of body-weight (Woolley, 1942). The growth rate of several other species of animals fed on purified diets is likewise increased when inositol is fed (see Woolley (1944) for bibliography).

Martin (1941) observed only slight alopecia in mice fed a diet deficient in inositol, while Jukes (1940) and Richardson, Hogan, Long and Itschner (1941) have raised rats to maturity on inositol-free diets. Part of the explanation probably lies in the fact that intestinal organisms may synthesise inositol (Woolley, 1940 c), although the presence of pantothenic acid is also essential for this process; further, pantothenic acid and inositol appear to be synergistic, as the symptoms of inositol-deficiency can appear in mice receiving inositol but not pantothenic acid (Scudi and Hamlin, 1942; Woolley, 1942). Synergism between these two vitamins has also been noted by Pavcek and Baum (1941), who found that inositol produced a dramatic response in the condition known as 'spectacled eye' in rats (Goldberger and Lillie, 1926; Oleson, Bird, Elvehjem and Hart, 1939) which Unna (1940) had shown could be improved by the administration of pantothenic acid.

The relationship between inositol and p-aminobenzoic acid has also received attention. Martin (1942) has reported that the latter, when fed to rats, caused a syndrome characterised by poor growth and changes in the fur, and that it was prevented by the administration of inositol. These two substances may also be concerned in lactation. Sure (1941a, b) reported that a rice bran and a liver extract added to a purified diet permitted normal lactation in rats which, although they grew and reproduced normally, failed to rear their young; subsequently he stated (Sure, 1943) that p-aminobenzoic acid was the active agent in his extracts, and, further, that inositol had a pronounced injurious influence, which was counteracted by p-aminobenzoic acid. On the other hand, Climenko and McChesney (1942) reported that, as judged by infant mortality, inositol had a beneficial and critical role in the maintenance of lactation in rats, and led some evidence to show that p-aminobenzoic acid may have supplemented its action.

Inositol appears to have some stimulating action on the motility of the gastro-intestinal tract (Anderson, 1916; Anderson and Bosworth, 1916; Martin, Thompson and de Carvajal-Forero, 1941; Bly, Heggeness and Nasset, 1943), but the mechanism of its action is not known. Nor is its intermediary metabolism fully understood; it appears to be an integral part of the enzyme pancreatic amylase (Williams, Schlenk and Eppright, 1944); and has also been found in phospholipids (Anderson and Roberts, 1930; Folch and Woolley, 1942). This latter fact may explain its well-recognised lipotropic

action, which has already been discussed (p. 96). However, it does not appear ever to have been associated with any defect in erythropoiesis, although the subject has received little study; Fenton, Cowgill, Stone and Justice (1950) recently reported that its absence from the diet of mice did not affect either their rate of growth or blood picture.

From the above review, it can be concluded that inositol is an essential nutrient for the mouse, although the fact that, in the presence of pantothenic acid, it can be synthesised by the intestinal flora means that it may not be an essential constituent of the diet. It is a lipotropic agent, and may also have a beneficial effect on the maintenance of lactation; but there is no evidence that it affects erythropoiesis. Its deficiency is manifested in impaired growth, and often - though not always - in a characteristic alopecia of the trunk. The mechanism of its action is not fully understood, but the presence of pantothenic acid appears to be necessary for the production of a maximum response to its administration; reports on the relationship between inositol and p-aminobenzoic acid are conflicting.

Thus, although there was no evidence that deficiency of inositol could have been responsible for the haematological effects produced by the calcium carbonate supplement to the Sherman B diet, it seemed not impossible that both the impaired rearing performance and the fatty livers might have resulted from such a cause. This, together with the fact that calcium carbonate could conceivably have induced a dietary deficiency of inositol, made me resolve to test the effects of adding a supplement of this substance to the high calcium carbonate diet. This was done in Experiment 7.

Iodine

The results of Experiment 4 indicated that the addition of calcium carbonate to the Sherman B diet for breeding mice may have resulted in hypothyroidism, and it was concluded that this condition may have been partly

responsible for the anaemia which regularly follows the feeding of such a diet.

In discussing the relationship between dietary calcium and thyroid activity (p. 149), it was seen that supplementation of the diet with calcium salts has been reported to reduce the levels of iodine in the blood and in the thyroid gland, and to increase the output of iodine in the urine; and it was pointed out that this may account for the hyperplastic goitre often associated with high intakes of calcium in the absence of adequate dietary iodine.

For these reasons, I decided to determine whether the addition of an iodide to a diet high in calcium carbonate would affect the level of blood haemoglobin. This also was done in Experiment 7.

EXPERIMENT 7

Experiment 7 really comprises two quite separate and distinct investigations; they were carried out concurrently, however, and will be described together for convenience.

The object of the experiment was to determine the effects of two substances - inositol and an iodide - when added to the Sherman B diet in the presence of a high level of calcium carbonate; for control purposes, the same supplements were also tested in the presence of a low level of calcium carbonate.

1. Construction of diets

The basal diets chosen were those used in Experiment 2, namely Diet 79 (low calcium carbonate) and Diet 81 (high calcium carbonate).

Inositol

The percentage of inositol supplement to be added to Diet 81 was calculated as the total amount contained in that quantity of phytic acid stoichiometrically equivalent to 1.40 parts of calcium carbonate, *i.e.* the difference in calcium carbonate content between 100 parts of Diet 79 and 100

parts of Diet 81. Thus, even if all the additional calcium in Diet 81 were to combine with phytate ions before inositol could be released, the use of this level of inositol supplement ensured that the amount of free inositol of dietary origin would be no less than that provided by Diet 79. In fact, of course, it was unlikely that all the calcium would combine in this way, and in any case the amount of additional dietary calcium doubtless exceeded the amount stoichiometrically equivalent to all the phytate in the diet;* Nevertheless, a certain amount of inositol is normally synthesised by the intestinal flora, and - although there were no positive grounds for believing that this process might have been interfered with - there seemed to be no harm in employing the full amount, as there is no evidence that inositol is toxic.

The calculation was made in the following way:

The inositol molecule is $C_6H_6(OH)_6$ (molecular weight 180.16) and forms phytic acid by union with ortho-phosphoric acid:

$$C_6H_6(OH)_6 + 6H_3PO_4 \rightleftharpoons C_6H_6(PO_4)_6H_{12} + 6H_2O.$$

According to Hoff-Jørgensen (1946), in the presence of calcium ions phytic acid forms penta-calcium phytate, $C_6H_6(PO_4)H_2Ca_5$. Thus 5 atoms of Ca (atomic weight = 40) are equivalent to 1 molecule of inositol (molecular weight = 180).

Thus, 200 g. calcium $\equiv$ 180 g. inositol.

Now, 1.4 g. of calcium carbonate contain 0.56 g. of calcium, and therefore the quantity of inositol required to balance this must be:

$$\frac{0.56 \times 180}{200} = 0.50 \text{ g.}$$

---

* All the phytate in the diet came from the wheat, 66 g. of which were contained in 107 g. of Diet 81. If the wheat contained 0.36% total phosphorus, and 85% of this was in the form of phytate, then 107 g. of Diet 81 contained 0.202 g. of phytate phosphorus. This is equivalent to 0.224 g. of calcium, whereas the additional calcium in 107 g. of Diet 81 amounted to 0.60 g.

The required level of inositol supplement* was thus 0.50%, and was achieved by adding 0.54 g. to each 107 g. of Diet 81; for the inositol-control diet, 0.54 g. was added to 105.5 g. of Diet 79. Each 100 g. of diet fed to the dams receiving inositol was further supplemented with 500 µg. of calcium pantothenate (i.e., a rate of 5 p.p.m.), as the pantothenate radicle appears to be necessary for inositol to exert its physiological effects.

Iodide

Sodium iodide was selected as a suitable soluble iodide. Hellwig (1934) found that a daily dose of 20 µg. of potassium iodide was sufficient to maintain a young rat, on a high calcium diet, in a euthyroid state - or at least prevented epithelial proliferation in the thyroid gland. If the requirement for the mouse were assumed to be half that of the rat, and were based on an average daily food intake of 10 g., 1 p.p.m. (i.e. 1 µg./g.) of potassium (or sodium) iodide would therefore be a sufficient supplement to the basal diet. However, the iodine content of both wheat and dried milk is usually of the order of only about 0.2 µg./g. - a rather low figure (Hellwig (1934) found that 2 µg. of potassium iodide per rat per day were insufficient to prevent hyperplastic goitre in rats receiving about 0.75 g. of calcium daily) - and consequently I decided to use a level of iodide supplement sufficient to ensure an adequate supply. The level chosen was 10 p.p.m. of iodide iodine, which is well within the minimal toxic dose (Simpson, B. W., personal communication).

This level could have been achieved by adding 1.24 mg. of sodium iodide to each 107 g. of Diet 81, but as this quantity was so small I decided to administer

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* Due care was taken to ensure that the supplement employed was the i-isomer (i.e. meso-inositol). Dr. P. C. Arni, of the Department of Protein and Carbohydrate Chemistry, Rowett Research Institute, kindly examined a sample at my request, and reported that the extent of its polarising activity was less than 1 per cent.

it in the water used for making up the dough. Instead of distilled water, therefore, the diet was made up with an aqueous solution of sodium iodide, 34.8 mg./litre; this was equivalent to 10 parts of iodine per million of dry diet. The same solution was used with Diet 79 to form a low calcium-high iodide control diet.

2. Design

Each part of the experiment, as we have seen, involved the comparison of two experimental diets with two basal diets. For economy and convenience the same animals were used as the basal diet controls for both parts of the experiment.

The two basal diets, Diets 79 and 81, had therefore each to be tested against similar diets containing inositol and sodium iodide respectively. As the effects of calcium carbonate were by now well established, there was no need to have within-litter comparisons between the high and low calcium carbonate levels. Thus, litters of three females each sufficed, and as sixteen such litters were available, the design consisted of two unrelated portions - one with a high and the other with a low level of calcium carbonate - each portion consisting of eight replications of three littermates receiving basal, inositol-supplemented and iodide-supplemented diets respectively (Text Table 67). The individuals of each litter were assigned to diet-groups by stratification according to individual weight (Text Table 68).

3. Procedure and methods

The sixteen litters, each of three females, were bred on stock diet in the usual way, and introduced to their appropriate experimental diets at seven weeks of age. One week later they were mated monogamously with males of similar age. The males were also littermates bred in the same way, each complete litter of females being mated with a complete litter of males. Brother-sister mating was avoided. The females were allowed to become pregnant for a second time before

TEXT TABLE 67.

Exp. 7. Design

	Low $CaCO_3$	High $CaCO_3$
Basal	(79)	(81)
Inositol		
NaI		

TEXT TABLE 68.

Exp. 7. Arrangement of littermates

Female litter no.	DIET		
	79	79 + Inositol	79 + KI
1	1	3	2
3	2	3	1
5	1	2	3
7	3	1	2
10	1	2	3
12	3	1	2
14	2	3	1
16	1	3	2

Female litter no.	DIET		
	79	79 + Inositol	79 + KI
2	3	2	1
4	1	2	3
6	2	1	3
8	3	2	1
9	3	2	1
11	1	2	3
13	3	2	1
15	2	1	3

the males were removed from the breeding cages.

The young mice were examined on weaning at 21 days of age. Haemoglobin examinations were conducted on four representatives of all litters. The median lobe of the liver was retained from one member of each first litter in diet-groups receiving inositol; and the thyroid gland was retained from a representative weanling in each first litter born of dams receiving sodium iodide. Both these procedures were also carried out on a representative of all litters from Diets 79 and 81. The blood haemoglobin concentration of the dams was estimated on the day that their second litters weaned, while with the sires this was done whenever the dams were seen to be pregnant for the second time.

Haemoglobin estimations were performed photocolorimetrically. Liver sections from first litters were prepared and stained for fat in the usual manner. Considerable difficulty was experienced in dissecting out the thyroid gland in 21 day-old mice; it is so small as to be scarcely distinguishable to the naked eye, but it can be clearly seen with the aid of a dissecting microscope. Nevertheless, it was found practically impossible to remove it from its close attachment to the thyroid cartilage of the larynx without severely damaging it. In a number of cases most of the gland was removed and an attempt made to weigh it; the wet weight, however, was considerably less than 1 mgm. in every instance, and I was forced to conclude that no reliable measurement of weight was possible.

The technique adopted to examine the thyroid glands histologically involved the dissection and removal of the entire larynx, together with the first few tracheal rings. The tissue (viewed under a dissecting microscope) was trimmed with a pair of fine scissors until the anterior and posterior edges of the thyroid gland were almost flush with the cut surface. After fixation, dehydration and clearing in the usual way, the tissue was embedded in paraffin

TEXT TABLE 69.

Exp. 7. Summary of results (unadjusted data)

Litter means	1st litters						2nd litters					
	Low $CaCO_3$			High $CaCO_3$			Low $CaCO_3$			High $CaCO_3$		
	79	79 + Inositol	79 + NaI	81	81 + Inositol	81 + NaI	79	79 + Inositol	79 + NaI	81	81 + Inositol	81 + NaI
No. born	8.9	9.1	9.8	8.3	8.3	8.5	5.1	8.6	8.9	5.5	7.1	5.4
No. weaned	5.3	7.3	7.8	3.6	2.8	2.6	2.5	7.8	7.1	2.4	4.6	2.3
Proportion weaned (%)	59	79	79	44	31	33	49	90	80	43	65	42
Total wt. weaned (g.)*	48.4	49.5	69.1	47.8	19.3	27.8	33.5	65.5	33.9	27.9	50.6	27.6
Mean wt./weanling (g.)*	7.3	6.9	8.1	6.6	5.5	5.9	10.1	8.7	8.3	9.3	7.9	7.7
Hb (g./100 ml.)*	2.99	3.34	4.71	2.75	1.94	1.82	4.26	3.58	3.55	2.89	2.49	3.05

Means	Dams						Sires					
Mating wt. (g.)	21.9	21.9	21.7	20.7	21.4	21.0	26.9	26.1	26.2	25.4	26.5	25.2
Final wt. (g.)	32.7	33.2	34.4	31.1	31.0	29.3	34.2	34.9	34.0	31.5	34.8	31.2
Gain in wt. (g.)	10.8	11.3	12.7	10.4	9.6	9.3	7.3	8.8	7.8	6.1	8.3	6.0
"M" period (days)	21.8	22.3	22.0	22.0	22.0	22.1	-	-	-	-	-	-
Period between litters (days)	29.0	35.0	33.1	33.0	34.8	28.4	-	-	-	-	-	-
Hb (g./100 ml.)	14.19	13.38	12.83	9.46	8.92	9.07	15.04	15.10	14.56	13.70	12.92	13.51

* Mean of 6 replications (low $CaCO_3$ diets), of 4 replications (high $CaCO_3$ diets, 1st litters), and of 5 replications (high $CaCO_3$ diets, 2nd litters).

wax in such a fashion that the microtome knife would cut transverse sections through it. Sections were stained by haemalum and eosin.

4. Results

All does bore two litters, but the rearing performance on the diets containing the higher level of calcium carbonate was poor. Of the twenty-four dams in the low calcium carbonate block, only two failed to rear their first litters, while two others failed to rear their second litters; but in the high calcium carbonate block, ten first litters and six second litters were not reared. This naturally affected the number of replications usable for analysis of the data.

A summary of the results is shown in Text Table 69. From this Table, it can readily be seen that the effect of the difference in rate of calcium carbonate supplementation is once more evident in every attribute; it must be remembered, of course, that this comparison is not a within-litter one, but nevertheless it is so marked as to leave little room for doubt that the higher level of supplement had produced its expected effect.

High calcium carbonate diets. Four first litter replications, and five second litter replications, were complete. As can be seen from the Table, with first litters there were no important differences in any attributes between the high calcium carbonate diets, except that on Diet 81 the total weight weaned was greater, and on analysis proved to be almost significantly greater, than that on the other diets ($P \doteqdot 0.05$). With second litters, the inositol-rich diet supported a rather better reproduction performance than the others, but there was no suggestion that differences were significant, and the individual weanlings were neither heavier nor less anaemic. There were no differences in any attribute with dams or sires.

TEXT TABLE 70.

Exp. 7. 1st litters. Liver fat. (Values assessed histologically, from 0-4)

Litter nos.	DIET			
	79	79 + Inositol	81	81 + Inositol
1/2	2	1	1	4
3/4	4	2	4	3
5/6	3	2	3	3
7/8	...	...	...	...
9/10	3	3	...	...
11/12	2	2	2	4
13/14	1	3	...	...
15/16	1	2	...	...
Mean	2.3	2.1	2.5	3.5

FIGURES 32-33

Exp. 7. Thyroid glands of weanlings

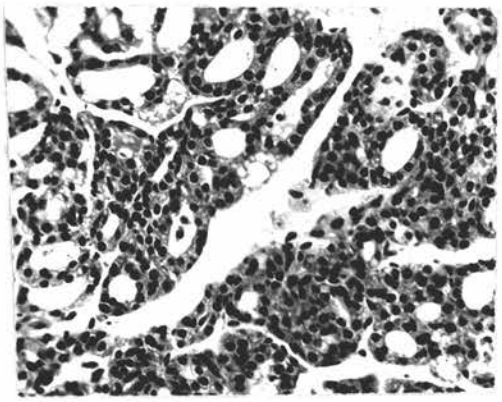

Fig. 32. Low calcium carbonate diet-group. (Haemalum and eosin; x 400)

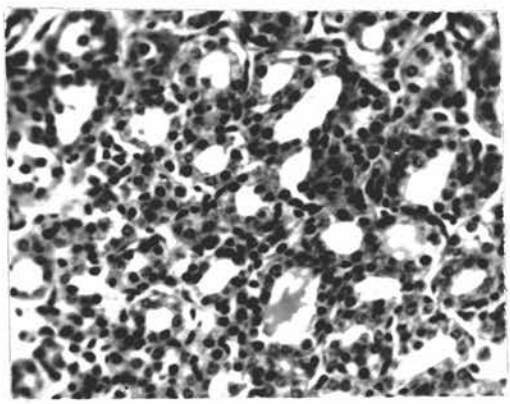

Fig. 33. High calcium carbonate diet-group. (Haemalum and eosin; x 400)

FIGURES 34-35

Exp. 7. Thyroid glands of weanlings

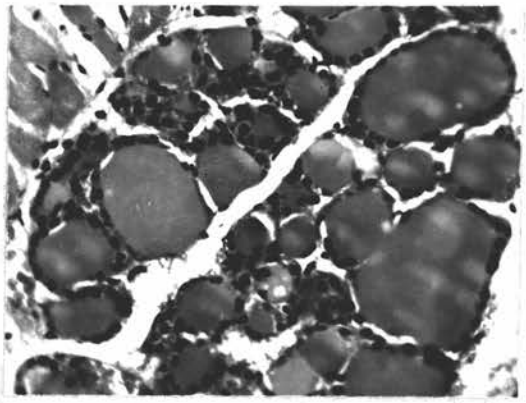

Fig. 34. Low calcium carbonate plus iodine diet-group.
(Haemalum and eosin; x 400)

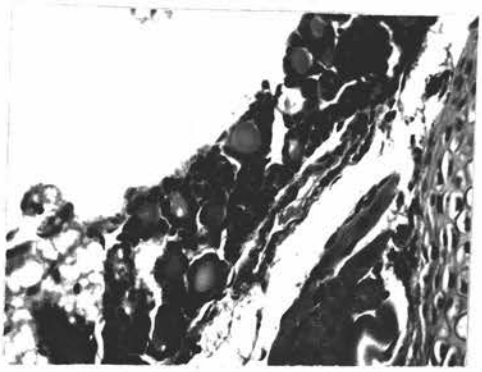

Fig. 35. High calcium carbonate plus iodine diet-group.
(Haemalum and eosin; x 400)

Low calcium carbonate diets. Six replications were complete with both first and second litters. With first litters, the iodide supplement appeared to be beneficial in the absence of the higher level of calcium carbonate, and a statistical analysis of the full data, after adjustment for missing observations, showed that the total weight weaned and the blood haemoglobin concentration (Hb) were both greater with the iodide supplement than without it ($P < 0.01, < 0.05$ respectively). With second litters, however, this was not so. The reproduction performance on Diet 79 was poor with both first and second litters, but the surviving weanlings compared favourably, especially in second litters, with those from the other diet-groups.

The inositol supplement did not affect first litters, although it supported a rather better reproduction performance with second litters; but in no case was any difference significant.

The supplements did not affect any attribute of the parent animals.

Liver fat. Examination of the frozen sections was carried out as in Experiment 2. The results are shown in Text Table 70, from which it is clear that the inositol supplement had no effect on liver fat. However, the Hb and liver fat values of all animals, irrespective of diet-group, proved to be inversely correlated ($r = -3.92 \pm 1.28$; $P < 0.01$). The mean Hb of animals with liver fat value 1 was 3.66; with value 2, it was 3.03; with value 3, it was 2.29; and with value 4 it was 1.70.

Thyroid glands. None of the thyroid glands examined was normal, but the abnormalities varied considerably in degree within diet-groups. The glands shown in Figs. 32-35, however, represent typical specimens from each diet-group.

Generally speaking, those from Diet 79 were hyperplastic, with many solid or narrow tubular acini, especially in the centre of the gland. The lumina were

frequently empty, but some contained scanty-pale-staining colloid. The acinar epithelial cells were columnar. The picture was therefore that of hyperplastic goitre.

The glands from Diet 81 were similar, but the hyperplasia was even more marked, with a notable hyperaemia; in some instances, only the acini at the periphery of the gland contained lumina.

The thyroid glands of animals from the iodine-rich diet-groups were different. On the low level of calcium carbonate, the acini were much larger and filled with deep-staining colloid, and the epithelial cells were flattened. In several instances, however, signs of hyperplasia persisted. On the high level of calcium carbonate evidence of hyperplasia was invariably seen, and the acini were small. Some of them were solid, but where lumina existed they usually contained dense colloid. The general picture in both cases was that of a colloid goitre with a variable amount of epithelial proliferation.

5. Discussion

The experiment showed that the meso-inositol supplement did not affect either reproduction performance or the visible fat in the livers of weanlings - nor, for that matter, did it affect haemoglobin concentration, although there had been no evidence to suggest that it might do so. This was true even although the diet contained adequate pantothenate, without which inositol has been said not to be capable of acting. Thus it can be concluded that the effects of dietary calcium carbonate are not attributable to inositol deficiency.

The histological examination of the thyroid glands produced distinct evidence that the basal diet (Diet 79) was goitrogenic, and suggested also that the addition to it of calcium carbonate merely intensified this property. The iodine supplement tended to reverse the hyperplastic changes in the thyroid glands and this may have accounted for the increased haemoglobin concentration

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PLEASE TURN OVER
TO p. 196

and the superior growth performance, which (with first litters) were supported by this supplement in the absence of the higher level of calcium carbonate. In the presence of the higher level, however, even this large dose of iodide failed to arrest completely the thyroid hyperplasia; while it diminished its severity, it did not at the same time affect the other attributes examined. It was also completely without effect on any attributes of the second litters and parent animals.

The highly significant correlation between liver fat and haemoglobin concentration confirms the results of previous experiments.

6. Conclusions

It can be concluded that, (1) the experiment produced no evidence to show that the effects of dietary calcium carbonate were attributable to inositol deficiency; and also that, (2) although the basal diet was goitrogenic, and calcium carbonate probably made it more so, there was no evidence to indicate that hypothyroidism resulting from iodine insufficiency was responsible for the effects of calcium carbonate on erythropoiesis and reproduction.

G. CALCIUM CARBONATE AND CHOLINE

In discussing the various causes of fatty livers, reference was made to the lipotropic action of choline. It was concluded that choline deficiency could have been responsible for the fatty changes seen in the livers of weanlings whose dams had been fed high levels of calcium carbonate.

On further study of the question, however, other considerations indicate that this was unlikely to have been the case. In the first place, choline deficiency - like other causes of 'dietary' fatty livers - is said usually to be accompanied by hypolipaemia, whereas in fact hyperlipaemia had been observed. Conceivably, however, an increase in plasma fat could have been due to a separate cause. In the second place, the diet must have contained large amounts of choline, since almost one-third of it consisted of dried milk - a rich source. Finally, there is no evident reason to suppose that a high level of dietary calcium carbonate will interfere with choline availability.

Nevertheless, this last point had never, to my knowledge, been put to experimental test, and it seemed worth while to do so at this stage. From such an experiment it would be possible to decide whether choline deficiency could be eliminated - or should be incriminated - as the cause of the liver lesions in the mice. The point was therefore tested in Experiment 8.

EXPERIMENT 8.

Procedure

The basal diet employed was Diet 69, which contains 2 per cent. of added calcium carbonate. To prepare the experimental diet, Diet 69 was supplemented with choline chloride at the rate of 1 mg. per g. - probably many times the requirement of the mouse.

When seven weeks of age, one mouse from each of six pairs of littermate females was assigned to Diet 69 and the other member of the pair to the experimental diet, the heavier members of each successive pair being allocated to the two diets alternately. One week later they were mated monogomously with pairs of littermate males of similar age, and these were removed from the breeding cages when the does were seen to be pregnant. Only one litter was bred from each doe.

Breeding records were maintained, and, on weaning at 21 days of age, a representative from each litter was examined. First its haemoglobin concentration was measured, and then, after it had been destroyed with ether, frozen sections stained with Scharlach R and haemalum were prepared from the median lobe of its liver. The amount of visible fat in each section was then assessed histologically.

Results

The results are shown in Text Table 71.

All twelve does bore litters, but one on Diet 69 devoured her young before they could be counted. The rearing performance was very poor on both diets, as was to be expected in view of their content of calcium carbonate; only seven litters were reared even in part, and only two sets of cousins survived.

The haemoglobin level of all surviving weanlings was very low, and it was clear that the addition of choline to the diet had not affected haemoglobin concentration in any way. The same was true of the liver fat values: all the livers were fatty, those from the high-choline group being equally as severely affected as the controls.

Conclusions

The addition of choline chloride to a diet containing a high level of calcium carbonate did not overcome the deleterious effects associated with this

TEXT TABLE 71.

Attribute \ Litter	DIET 69								DIET 69 + Choline							
	A	B	C	D	E	F	Total	Mean	A	B	C	D	E	F	Total	Mean
No. born	(?)	7	7	8	10	11	43	8.6	(7)	7	9	8	8	10	42	8.4
No. weaned	0	0	0	4	6	9	19	3.2	0	1	2	6	6	0	15	2.5
Survival rate (%)	...	...	...	...	...	...	...	44	...	...	...	...	...	...	...	36
Total wt. weaned (g.)	0.0	0.0	0.0	23.1	35.4	65.6	124.1	20.7	0.0	5.2	15.8	27.0	30.3	0.0	78.3	13.1
Mean wt. weaned (g.)	...	...	...	...	...	...	...	6.5	...	...	...	...	...	...	...	5.2
Hb (g./100 ml.)	-	-	-	3.26	3.70	4.00	10.96	3.65	-	2.22	5.33	1.63	3.26	-	12.44	3.11
Liver fat (units)	-	-	-	2	2	2	6	2.0	-	4	3	3	2	-	12	3.0

supplement, and, in particular, did not affect the severity of the fatty change in the livers of weanlings. It was therefore concluded that deficiency of choline was not the cause of the fatty livers.

H. FURTHER STUDIES ON CALCIUM CARBONATE AND IRON

In Experiment 2 and succeeding experiments it has been shown that when calcium carbonate is added to the Sherman B diet (which is marginally deficient in iron) and fed to breeding mice, anaemia develops in both the dams and their young. It has also been shown that an iron supplement will raise the haemoglobin concentration of such animals, but on the other hand it has not been proved that calcium carbonate actually interferes with the availability of dietary iron.

Indeed, from the results of Experiment 2 it is clear that calcium carbonate does not completely prevent the utilisation of iron. It may, however, do so partially, in which case it would be expected that severe anaemia would not follow the addition of calcium carbonate to a diet rich in iron, as under these circumstances sufficient iron would still be available for haemoglobin synthesis. Accordingly, I decided to test this point by experiment (Experiment 9).

EXPERIMENT 9

1. Construction of diets

As an iron-rich basal diet, a commercial preparation of 'National Pig Food no. 1 for sows and weaners' was selected. This was identical in composition with Diet 4 of Howie and Porter (1950), and differed only in being fed in the form of a dough instead of as a compressed cube. It was reputed to contain: ground barley 28, fine bran 14, manioc meal 5.8, extracted palm meal 8.3, palm cake meal 5.8, white-fish meal 5.8, meat-and-bone meal 3.0, ground maize 16.5, flaked maize 3.0, maize-gluten meal 8.3, cod-liver oil 0.6, ground limestone 0.5 and sodium chloride 0.4%. Spectrographic analysis* showed that the diet as fed contained

*Kindly carried out for me by Dr. R. L. Mitchell, Macaulay Institute for Soil Research, Aberdeen.

135.3 p.p.m. of Fe, or about 100 p.p.m. more than Diet 79. Chemical analysis* showed that its calcium (as Ca) and phosphorus (as P) contents were respectively 0.98% and 0.69% giving a Ca : P ratio of 1.42 : 1.

The calcium carbonate supplement added to this diet was, as before, at the rate of 2 per cent. This raised the Ca : P ratio to 2.58 : 1. Before mating for second litters, however, it was increased to 6 per cent., giving a Ca: P ratio as high as 4.90 : 1.

2. Design

Three sets of four littermate females were employed, and each set was divided into two groups of two at random, so that each diet-group contained six mice. Larger groups might have been advantageous, but the necessary numbers of mice were not available at the time.

3. Procedure and methods

The mice employed were bred in the usual way from the stock colony, the litters of females being divided into diet-groups when seven weeks of age and immediately introduced to their experimental diets. One week later they were mated monogamously with males of similar age, the males being removed when the females were seen to be pregnant.

Records of reproduction performance were kept, and the young mice were weighed on weaning at 21 days old, at which stage also haemoglobin estimations (by Haldane's method) were made on four representatives of each litter.

The males were then replaced in the breeding cages, and the very high calcium carbonate diet introduced during the breeding and rearing of second litters; the latter were similarly examined at 21 days of age, as were their dams on the same day.

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* Kindly carried out for me by Mr. J. Davidson, Rowett Research Institute.

TEXT TABLE 72.

Exp. 9. Summary of results.

	Litter No.	No. born		No. weaned		Survival rate (%)		Total wt. weaned (g.)		Mean wt./ weanling (g.)		Litters' Hb (g./100 ml.)		Dams' Hb (g./100 ml.)	
		B*	S*	B	S	B	S	B	S	B	S	B	S	B	S
1st litters	A	7	10	7	10	-	-	56.6	77.0	8.1	7.7	6.96	5.48	-	-
		10	8	10	8	-	-	80.8	72.0	8.1	9.0	6.66	6.51	-	-
	B	11	2	11	2	-	-	85.1	24.1	7.7	12.0	7.70	7.25	-	-
		6	7	0	7	-	-	0.0	65.9	-	9.4	-	6.51	-	-
	C	9	7	9	7	-	-	84.7	53.7	9.4	7.7	9.84	8.81	-	-
		9	7	9	2	-	-	93.4	12.9	10.4	6.5	8.51	3.85	-	-
	MEAN	8.7	6.8	7.7	6.0	88.5	87.8	66.8	50.9	8.7	8.5	7.93	6.40	-	-
2nd litters	A	10	0	7	0	-	-	68.1	0.0	9.7	-	6.22	-	12.00	-
		0	8	0	7	-	-	0.0	58.2	-	8.3	-	4.00	-	12.28
	B	0	6	0	3	-	-	0.0	23.0	-	7.7	-	2.00	-	7.40
		4	0	4	0	-	-	43.4	0.0	10.8	-	10.43	-	14.80	-
	C	7	7	6	6	-	-	62.7	56.5	10.4	9.4	10.51	5.92	13.47	12.28
		11	5	11	5	-	-	89.2	41.6	8.1	8.3	9.62	2.81	13.91	11.54
	MEAN	5.3	4.0	4.7	3.5	87.5	79.2	65.9	44.8	9.4	8.5	9.19	3.69	13.54	10.66

* B = basal diet: S = supplemented diet.

4. Results

A summary of the results is presented in Text Table 72.

As the Table shows, all the twelve does bore first litters, although one (on the basal diet) failed to rear any of its young and another (on the supplemented diet) lost part of its litter. The reproduction and rearing performance was thus quite good.

Although with first litters the basal diet gave a superior result in every attribute examined, the differences were not very large and none proved significant after statistical analysis. Clearly the calcium carbonate had been less (if at all) deleterious when added to this high-iron diet than when added to the marginally iron-deficient Sherman B diet.

Two animals in each group failed to reproduce a second time, and consequently second litters from only four pairs of littermates were available for comparison. Differences were clearly larger, however, and again invariably favoured the basal diet-group. The difference in mean haemoglobin level, even with only four degrees of freedom, proved to be significant ($P < 0.05$); and the difference in total weight weaned also appeared to be real ($P < 0.10$).

With the dams, the difference in mean haemoglobin concentration was also large, although it did not reach significance.

5. Discussion and conclusions

The experiment might have given even more clear-cut results if the diet-groups had been larger; nevertheless, it clearly demonstrated that if the diet is rich in iron, a moderate supplement of calcium carbonate is not markedly deleterious although a very large supplement will still affect reproduction and induce profound anaemia. The results of the experiment thus support the hypothesis that calcium carbonate partially interferes with iron availability.

Incidentally, the experiment has shown also that iron can be satisfactorily

utilised even if the Ca : P ratio of the diet is high; the basal diet, which supported a high haemoglobin concentration, had itself a ratio as high as 1.4 : 1, and the 2% supplement of calcium carbonate, which scarcely affected the haemoglobin level, raised the Ca : P ratio of the diet to 2.58 : 1. This ratio is higher than those of Diets 69 and 81, both of which have been seen to be strongly anaemigenic. Thus, if the level of iron in the diet is high, neither a wide Ca : P ratio nor a large absolute amount of calcium in the diet can entirely prevent its utilisation for the synthesis of haemoglobin.

I. CALCIUM CARBONATE IN THE DIET OF OTHER TYPES OF ANIMALS

The experiments so far described were all conducted on Swiss albino mice. As species differences and even strain differences are frequently encountered in biological work, small-scale trials were carried out to test the effects of dietary calcium carbonate on three other strains of mice ('Black' (C57), 'Hybrid' and 'Silver'), and also on rats.

EXPERIMENT 10 (Mice)

1. Procedure and methods

The diets used were Diet 79 (which contains 0.5% of added calcium carbonate) and Diet 81 (which contains 2% of added calcium carbonate) as used in previous experiments.

Two litters of each variety of mouse were employed, one litter containing at least four females, the other containing at least four males. The actual number of animals available for each strain is shown in Text Table 73. Both males and females, when seven weeks old, were assigned at random to the two diets, and were mated together one week later. Thus all matings were between littermates. Monogamous mating was not possible with all the 'Hybrid' and 'Silver' animals, as there were insufficient littermate males of these varieties; in each case, therefore, one male on each diet was mated with two females, the latter being removed to individual cages when seen to be pregnant. Two litters were bred from each dam.

In other respects, the procedure employed followed that described for previous experiments, as outlined in the Technical Appendix (p. 242). The only estimation made on the blood was haemoglobin concentration, which was done by Haldane's method.

TEXT TABLE 73.

Exp.10. Table of Means.

Attribute	Class of Animal	Black (C57)		Hybrid		Silver	
		Diet 79	Diet 81	Diet 79	Diet 81	Diet 79	Diet 81
		Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
No. of experimental animals	Dams	2	2	4	3	3	3
	Sires	2	2	3	2	2	2
No. born/litter	1st litters	7.5	8.0	10.8	9.7	9.3	6.3
	2nd litters	7.0	4.0	10.0	7.0	6.7	6.7
No. weaned/litter born	1st litters	7.0	7.0	7.5	5.0	2.3	2.3
	2nd litters	3.5	1.0	10.0	4.5	3.3	4.3
Total wt. weaned/litter born (g.)	1st litters	56.0	40.0	72.1	68.1	22.0	19.1
	2nd litters	28.7	5.6	92.8	31.4	34.8	39.5
Mean wt./weanling (g.)	1st litters	8.0	5.8	9.7	9.1	9.4	8.2
	2nd litters	8.2	5.6	10.2	7.0	11.0	9.6
Gain in wt. (g.)	Dams	9.7	9.8	14.6	10.4	6.2	8.0
	Sires	8.8	13.1	7.2	6.6	17.5	12.8
Hb (g./100 ml.)	1st litters	5.68	2.87	4.51	3.05	4.14	2.81
	2nd litters	8.15	1.92	4.57	1.85	7.53	3.02
	Dams	13.17	11.69	13.54	11.35	13.07	11.10
	Sires	11.40	12.14	12.28	12.43	13.76	14.06

TEXT TABLE 74.

Exp.10. Hb (g./100 ml.).

Strain	Black		Hybrid		Silver	
Class \ Diet	79	81	79	81	79	81
Litter-means (1st litters)	6.88 4.51	2.74 3.00	3.60 5.33 4.41 4.74	2.74 3.37	4.14	2.81
Mean	5.70	2.87	4.51	3.06	4.14	2.81
Litter-means (2nd litters)	8.24 8.07	1.92	4.29 4.78 4.66	1.85	8.63 6.44	3.00 3.03
Mean	8.15	1.92	4.57	1.85	7.53	3.02
Dams	13.62 12.73	10.95 12.43	15.10 13.32 12.14 13.62	12.43 11.69 9.92	15.84 13.76 9.62	12.43 13.32 7.55
Mean	13.17	11.69	13.54	11.35	13.07	11.10
Sires	10.95 11.84	12.58 11.69	12.73 13.02	11.84 13.02	13.32 14.21	13.91 14.21
Mean	11.40	12.14	12.14	12.43	13.76	14.06

2. Results and discussion

A summary of the results is given in Text Table 73.

The reproduction performance was rather poor, especially with the 'Silver' strain. Certainly, all but one doe bore two litters (the exception being a 'Black' doe on Diet 81, which produced only one); but five of the seventeen first litters, and also five of the sixteen second litters, were not reared. Of these ten litters, six (three in each set) were bred on Diet 81.

With so few animals involved, statistical analysis of the reproduction and growth attributes shown in Text Table 73 was out of the question, but it can be seen that Diet 79 again held the advantage in most instances. All that can be said of these results is that the size of the differences more or less conformed with expectations, and that their direction also caused no surprise.

Differences in haemoglobin concentration were generally quite large; with the sires, however, they were clearly unimportant. Text Table 74 shows the full results of the Hb estimations. With these results also, statistical analysis would obviously have yielded no useful information, but inspection of the figures indicates that calcium carbonate had a markedly negative effect on blood haemoglobin concentration in both dams and weanlings, but particularly in the latter. Stated in the most conservative way, the results of the experiment offer no reason to doubt that the higher level of supplement induced anaemia in these three strains, which behaved in this respect in the same way as the albino mice.

EXPERIMENT 11 (Rats)

1. Procedure and methods

(see also Technical Appendix, p. 247)

The diets used in this, as in the previous, experiment were Diets 79 and 81. Three quartets of female littermate rats, reared on stock diet, were randomised

between the two dietary treatments, with two animals from each quartet assigned to each diet. They were introduced to their experimental diets when twelve weeks of age, and one week later were mated with two littermate males - one male to each dietary group of six females.

The females when pregnant were removed to individual cages, where their litters were born. The litters were counted and weighed at birth, but at three days old were reduced to a maximum of eight pups, any in excess of that number being discarded. The young rats were weaned at 24 days of age, and at that time their blood haemoglobin concentration was determined by Haldane's method.

Three days later, each dam was returned to the mating cage for re-mating, and the same procedure as before was followed. Haemoglobin estimations were made on the dams at the time their second litters reached weaning age.

2. Results and discussion

The results given by first litters are summarised in Text Table 75(a), column (i). All the does on Diet 79 and all but one on Diet 81 bore first litters; but two dams on Diet 79 failed to rear their young, all of which were either stillborn or were destroyed by their dams at birth. At birth, pups from Diet 79 mothers were slightly fewer in number but in general were rather heavier than their cousins from diet 81 mothers, and they still retained this superiority in weight when weaned. Differences were very small, however, and must be discounted for that reason. The differences in haemoglobin concentration were also small, but again tended to favour Diet 79 (see also Text Table 77).

The second litter breeding performance was very poor. Five litters were born on Diet 81 and all were reared, but only three litters were born from dams on Diet 79, and two of them were destroyed at birth by their mothers.

TEXT TABLE 75.

Exps. 11 & 12. Table of Means.

(a) Litters.

	(i) Exp.11 -1st litters		(ii) Exp.12 -1st litters		(iii) Exp.12 -2nd litters		(iv) Expts.11 & 12 (combined)	
	-	$CaCO_3$	-	$CaCO_3$	-	$CaCO_3$	-	$CaCO_3$
No. of litters born (total)	6	5	8	7	8	7	22	19
No. of litters reared (total)	4	5	7	6	8	7	19	18
No. of pups born alive per litter	8.0	9.4	11.1	9.9	8.9	10.3	9.5	9.8
Mean wt. of pups born alive (g.)	5.4	5.1	5.5	5.4	6.1	6.4	5.67	5.69
Mean wt. per weanling (g.)	35.2	33.9	38.3	35.4	49.3	43.2	42.32	37.71
Mean Hb (g./100 ml.)	6.85	6.02	8.35	7.50	6.66	5.96	7.73	6.53

(b) Dams.

	Exp. 12	
	-	$CaCO_3$
Mean wt. (g.)	263.8	260.6
Mean Hb (g./100 ml.)	13.62	13.91

Consequently the results are too scanty to deserve serious consideration. The only litter reared on Diet 79 had a mean Hb of 8.58 g./100 ml., and its cousins on Diet 81 had a mean Hb of 8.89 g./100 ml. - a negligible difference. The mean Hb of the three dams on Diet 79 which bore second litters was 13.72 g./100 ml., and that of the corresponding five dams on Diet 81 was 12.14 g./100 ml. The difference between the two is, however, quite meaningless, as within-group differences were much greater.

EXPERIMENT 12 (Rats)

1. Procedure and methods

This experiment was almost a repetition of Experiment 11. This time, however, Diets 2 and 69 were employed instead of Diets 79 and 81, with the object of increasing the disparity between the diets as regards their levels of calcium carbonate supplement. Another difference was that eight pairs of female littermate rats were used, instead of three quartets. The females were mated with two pairs of littermate males, each male being placed with four females, and its brother with their sisters.

2. Results

A summary of the results is presented in Text Table 75(a), columns (ii) and (iii), and (b).

Except for one doe on Diet 69, which proved to be completely infertile, all does bore two litters. However, one dam on each diet failed to rear her first litter, and another dam on Diet 69 destroyed her second litter at birth. None of these four animals were littermates and consequently, although the rearing performance of the remaining dams was fairly satisfactory, the balance of the experiment was to some extent disturbed. This proved relatively unimportant, however, as it was clear that all differences were again small.

TEXT TABLE 76.

Expts. 11 and 12. Mean weight per weanling (g.).

(Littermate dams only, weaning equal or nearly equal numbers of pups)

		-	$CaCO_3$
Exp. 11	1st litters	34.0 33.6 29.6	32.5 30.8[x] 32.9
Exp. 12	1st litters	29.8 27.0 42.3	33.5 33.4 35.7
	2nd litters	48.0 50.2 49.3 63.6	41.6 41.7 41.7 51.5
Overall Mean		40.7	37.5

[x] Average of two (31.2 and 30.3).

Further, the reproduction data were inconsistent, and obviously there were no important dietary effects.

The differences between diets with weanlings' mean weight and with their haemoglobin concentration were also small, but more consistent, and were in the same direction as the differences with the first litters in the previous experiment. When the data from the two experiments were combined (column (iv) of Text Table 75(a)), it seemed possible that there might be real differences with these two attributes. Consequently, the individual litter-means were compared in every case in which two littermate dams had both reared their litters. (In the case of 'Mean wt./weanling', comparisons were restricted to those instances where equal or nearly equal numbers of young had been reared, because of the obvious interaction between the number in the litter and the weights of the individual weanlings.) These comparisons are shown in Text Table 76.

From this Table it is clear that there was no consistent dietary effect, at least with first litters, on the weights of weanlings; with second litters, it is possible that longer experience of the diets may have resulted in a slight relative advantage being gained by the non-supplemented group. On the other hand, thirteen of the fourteen differences in haemoglobin concentration were in the same direction (Text Table 77). When analysed statistically these differences proved highly significant, and indicated that the calcium carbonate supplement fed to the mothers had reduced the haemoglobin concentration of their offspring at weaning age ($P < 0.02$). This effect, though significant, was nevertheless small, averaging only 0.82 g./100 ml. (or about 5½% Haldane). Thus although the effect of calcium carbonate on the blood haemoglobin concentration of weanlings proved to be the same with rats as with mice, it was very much less in degree. On the other hand, the supplement did not affect either the weights or haemoglobin concentration of the dams (Text Table 75 (b)).

TEXT TABLE 77.

Exps. 11 and 12. Mean Hb (g./100 ml.) of weanlings from littermate dams.

		-	$CaCO_3$
Exp. 11	1st litters	6.72	5.09
		6.45	6.57
		7.52	5.95
Exp. 12	1st litters	9.37	7.18
		7.10	9.03
		8.36	6.36
		6.96	6.51
		8.44	7.37
	2nd litters	7.25	6.22
		6.36	6.07
		5.92	4.59
		7.25	6.22
		6.73	6.66
		6.96	6.07
Overall Mean		7.24	6.42

Standard error of the difference between the overall means = $\pm$ 0.280.

3. Discussion

These experiments indicate that while there is probably no fundamental difference between the two species - rats and mice - in the nature of their response to a dietary supplement of calcium carbonate, yet rats have a much greater resistance to the effects of the supplement, or at least do not so readily show symptoms of the disturbance. Without exact knowledge of the method by which calcium carbonate interferes with iron utilisation for erythropoiesis, the reason for this must remain uncertain.

It may simply be a question of the amount of iron carried in the body stores; the body iron content is always very much lower in normal mice than in normal rats (Widdowson and McCance, 1948), although it increases at a faster rate between birth and weaning (Spray, 1950), and in the event of there being only a limited supply available from the dams, a deficiency would therefore be expected to be more obvious in weaning mice than in weaning rats. On the other hand, there may well be some more subtle difference between the two species in their ability to utilise iron in the presence of dietary calcium carbonate.

J. SERUM INORGANIC PHOSPHATE AND CALCIUM

In view of the profound effects which resulted in Experiments 1 and 2 from supplementation of the basal diet with calcium carbonate, the status of the blood plasma in regard to calcium and inorganic phosphate became of interest.

It is well-known that a high level of dietary calcium can induce a deficiency of phosphate. The calcium combines with phosphate in the alimentary tract, forming calcium phosphates and being excreted as such. Further, if the phosphate in the diet is insufficient to meet all the demands of the calcium, more phosphate is withdrawn from the body's reserves into the gut. This process results in decalcification of bone, and, if severe, will be manifested in hypophosphataemia.

It has been shown in Experiment 2 that the effects of the calcium carbonate supplement were not abolished - and indeed were scarcely affected - by the further addition to the diet of a phosphate supplement, sufficient in amount to restore the Ca : P ratio of the diet to its original value. The question now is whether or not this quantity of phosphate was sufficient to bind all the additional calcium. It cannot be stated precisely how much phosphate is required to equilibrate a given amount of dietary calcium, because the relative proportions of the various calcium phosphates excreted in the faeces are not constant. In the main, however, these salts are the tri-basic and di-basic orthophosphates of calcium ($Ca_3(PO_4)_2$ and $CaHPO_4$). With the tri-basic salt, two phosphate ions (each containing one atom of phosphorus) balance three atoms of calcium, while with the di-basic salt the proportion is one to one; the stoichiometric equivalents of calcium (as Ca) and phosphate phosphorus (as P) are therefore 60 : 31 and 40 : 31 respectively. Thus in either case a given weight of calcium can be balanced by a considerably smaller weight of phosphate

phosphorus. In order to restore the Ca : P ratio of the diet (originally practically unity) the quantity of phosphate phosphorus added had been approximately equal in weight to the weight of the added calcium (0.57% and 0.60% respectively), so that there should have been no need to call upon reserves of phosphate from the body, and no hypophosphataemia should have ensued.

1. SERUM INORGANIC PHOSPHATE

It was to test the above reasoning that estimations of serum inorganic phosphate were made on the serum of individual dams and on samples of serum pooled from all the weanlings in each second litter of Experiment 2.

1. Procedure and methods

Serum was prepared as described on p. 52, the chemical determination being made on the same day; the technical methods employed are described in the Technical Appendix (p. 260).

2. Results

The results are summarised in Text Table 78. A number of dams did not yield satisfactory samples of serum - either the quantity was too small or haemolysis was excessive. Consequently many of the estimations could not be made in duplicate, or could not be made at all, and in view of this the results were very incomplete and were not subjected to statistical analysis. However, it is evident that calcium carbonate did not induce a hypophosphataemia, although the diet-group means suggest that it may have had a depressing effect in the presence of phosphate, which, as might be expected, appeared to increase the value slightly.

The serum samples obtained from the litters were larger and clearer than

TEXT TABLE 78.

Exp. 2. Serum inorganic phosphate (mg./100 ml.). Diet-group means

Supplement	Dams		Litters	
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
-	7.5	7.1	11.8	11.5
Fe	7.4	7.2	10.8	11.0
P	8.6	7.8	12.1	10.8
Fe + P	9.5	8.1	12.9	11.2
Standard error of the difference between means each formed from:				
4 diet-groups	-		± 0.44	
2 diet-groups	-		± 0.62	
1 diet-group	-		± 0.87	
Overall effects of:				
$CaCO_3$	- 0.7		- 0.77	
Fe	+ 0.3		- 0.08	
P	+ 1.2		+ 0.48	
Interaction $CaCO_3$ x P	- 0.4		- 0.73	

those from the dams, and consequently the observations were more complete. Statistical analysis of the data did not reveal any significant effects or interactions, but the general pattern of the results is remarkably similar to that given by the dams. The calcium carbonate effect was again negative, and was quite large; but, as the interaction shows, it was produced almost entirely by the groups containing phosphate. The overall phosphate effect was positive and also fairly large; indeed, in the absence of calcium carbonate the phosphate effect approached significance ($+1.2 \pm 0.62$; $t = 2.03$ when $P = 0.05$).

Thus if the phosphate supplement raised the serum inorganic phosphate level (the evidence falls just short of proof), the additional presence of calcium carbonate apparently abolished this effect; but calcium carbonate alone did not materially lower the value. It may therefore be concluded that the effects of the calcium carbonate supplement on haematological and other attributes in Experiment 2 were not associated with hypophosphataemia.

2. SERUM CALCIUM

It is well-known that, in health, the concentration of calcium in the blood plasma is maintained at a remarkably constant level, and that even moderate fluctuations in this value result in disorders which are usually recognisable clinically. There had been no clinical evidence of hypercalcaemia or hypocalcaemia in mice from the high calcium carbonate groups, but it was nevertheless decided to check this point by comparing the serum calcium concentration of these animals with that of the controls on low calcium carbonate diets.

1. Procedure and methods

As each estimation required 2 ml. of serum, it was not possible to collect

TEXT TABLE 79.

Various exps. Serum calcium (mg./100 ml.).

Sample	Reading		Corrected reading	
Blank (1)	0.03	0.03	-	Mean
Blank (2)	0.03		-	
Diet 79 (1)	0.96		1.93	1.00
Diet 79 (2)	1.10		1.07	
Diet 81 (1)	1.03		1.00	0.97
Diet 81 (2)	0.96		0.93	

Normality of stock permanganate solution was 0.09929 $\overline{N}$

Therefore, serum Ca is:

Diet 79 ..... 9.9 mg./100 ml.

Diet 81 ..... 9.6 mg./100 ml.

sufficient blood to prepare suitable quantities of serum from dams. However, by pooling serum from litters on various experiments, but all of whose mothers had been fed Diet 79 or Diet 81, sufficient serum was obtained to make duplicate estimations for each diet. Details of the method of estimation employed are given in the Technical Appendix (p. 259).

2. Results

The results are shown in Text Table 79. The values obtained for both diets fall within the normal range, and there is obviously no important difference between them.

It may therefore be concluded that the calcium carbonate supplement had had no effect on the calcium content of the serum.

PART III

GENERAL DISCUSSION AND RÉSUMÉ

PART III

A. GENERAL DISCUSSION

The first two experiments described in this thesis showed that when calcium carbonate was added to the Sherman B diet, and fed to breeding mice, both dams and litters developed hypochromic microcytic anaemia. The supplement also impaired the rearing performance, and pathological features in the young animals included stunted growth, hypertrophy of the heart, fatty changes in the liver, hyperlipaemia, and atrophy of the thymus gland.

Examination of the literature showed that at one time or another such lesions or abnormalities have all been associated with anaemia, and it thus seemed probable that in these experiments they were not primary effects of the diet but arose secondarily to the anaemia. After discussion, however, it was concluded that although anaemia appeared to be their likeliest cause, certain other factors could not be excluded as alternative or additional causes. These factors have now all been studied experimentally, and it has been shown that none of them could have played any part in the aetiology of the syndrome. Consequently it must be assumed that the original hypothesis was a correct one, and that, of the observations described, only the anaemia was a primary effect of the calcium carbonate supplement. This view is also supported by the fact that positive correlations have been established between the degree of the anaemia and the severity of some, at least, of the other findings, including the liver lesions, the hyperlipaemia and the cardiac hypertrophy, these correlations being independent of diet.

The main factor concerned in causing the anaemia was undoubtedly a deficiency of iron. This view was first put forward tentatively in a previous discussion, after it had been demonstrated in Experiment 2 that the anaemia was hypochromic and microcytic and that improvement in every respect followed the

administration of a supplement of ferric citrate. At that time, however, it was pointed out that this evidence by itself was insufficient to prove that calcium carbonate had interfered with the utilisation of iron, as improvement had followed iron administration whether or not the diet contained a high level of calcium carbonate. Indeed, it was made clear that the utilisation of iron could not have been completely inhibited by calcium carbonate, otherwise no improvement would have been observed in its presence. But, on the other hand, it was also shown that because the improvement seen in the absence of calcium carbonate was no greater than that seen in its presence, it did not follow that partial interference could not have occurred; it was pointed out that the effect of the iron in low calcium carbonate diets would be limited by the 'room for improvement' principle, while fuller utilisation of all the available iron in high calcium carbonate diets would be encouraged by the very fact of iron deficiency itself.

In subsequent experiments, however, additional and more conclusive evidence has been forthcoming to support the suggestion that dietary calcium carbonate interferes with iron utilisation. In the first place, the general pattern of the Fe x $CaCO_3$ interaction in Experiment 4 conformed with this hypothesis: with first litters, which showed the most 'room for improvement', the effect of an 'Analar' iron salt in the absence of calcium carbonate was much greater than that in its presence; and this difference with first litters was greater than the corresponding differences with second litters, dams and sires, whose haemoglobin levels in the control diet-groups were much nearer the physiological optimum. Further confirmation was obtained from Experiment 9, with the finding that when the diet was rich in iron the addition of a moderate calcium carbonate supplement was not followed by anaemia, although anaemia did supervene when the rate of supplementation was greatly increased.

It has also been possible to exclude the possibility that certain other

known causes of hypochromic microcytic anaemia - such as copper deficiency and pyridoxin deficiency - might have been concerned, but at the same time it was found that dietary supplements of cobalt sulphate and desiccated thyroid gland both appeared to relieve the anaemia. Consequently, although there can be little doubt that iron deficiency was the chief cause of the anaemia, cobalt deficiency and hypothyroidism must be considered as possible subsidiary causes.

The possibility that an induced cobalt deficiency played a part in the causation of the anaemia rests, however, on very slender evidence. It is true that a cobalt supplement in the diet was seen to increase the haemoglobin concentration of the dams and their young, and also that its effects were less marked in the presence than in the absence of calcium carbonate; but these results by no means prove that the dyshaemopoiesis resulting from dietary calcium carbonate should be attributed, even partly, to a deficiency of cobalt, as it is well recognised that the administration of even very small doses of cobalt will cause polycythaemia accompanied by an abnormally high haemoglobin level. If traces of cobalt were contained in the ferric citrate preparation used in Experiment 2, it is certainly possible that the positive response to the administration of the preparation could have resulted partly from this cause; but it is very significant that a supplement of 'Analar' ferrous sulphate - the purest form of iron available - was also effective in raising the haemoglobin level. It should also be borne in mind that, despite many attempts to do so, anaemia in non-ruminants caused by cobalt deficiency has never been satisfactorily demonstrated (and even in ruminants it is at least questionable whether cobalt deficiency affects erythropoiesis directly). It must be concluded that the balance of the evidence is certainly against the likelihood that cobalt played a major part in the process.

Further work will have to be done before this question can be decided. The problem might be solved by making use of a chemically pure iron salt similar

to that developed by Elvehjem and his colleagues for their work on copper metabolism (see p. 131). If such a preparation were to be found equally as effective as crude ferric citrate of an 'Analar' iron salt the importance of minute traces of cobalt could be discounted. This question remains for the future, but for the present it may be said that the observation that the effects of cobalt are interfered with by the presence of dietary calcium carbonate is one that may prove to be of practical importance, particularly if it can be confirmed in ruminants.

The suggestion that hypothyroidism played a part in the causation of the anaemia is based on firmer ground. Anaemia has frequently been associated with deficiency of the thyroid hormone, and that salts of calcium are goitrogenic is also not a new observation - indeed it has several times been shown that when a diet contains a high level of calcium carbonate a high iodine intake is necessary to prevent hyperplasia of the thyroid gland. The present work confirmed these findings, and also showed that the administration of desiccated thyroid (which contains thyroxin) was particularly effective in raising the haemoglobin concentration when the diet contained calcium carbonate. In contrast, however, the administration of a high level of iodide with calcium carbonate failed to alleviate the anaemia, although in the absence of calcium carbonate it succeeded, at least with first litters, in raising the haemoglobin level in the same way as the desiccated thyroid had done.

Thus in the presence of calcium carbonate thyroid hormone succeeded, but iodide failed, to raise the haemoglobin concentration. The iodide was not entirely without effect, however, for even in the presence of calcium carbonate it caused the colloid in the thyroid gland to become increased; some, at least, of the iodide must therefore have been absorbed. The goitrogenic action of calcium carbonate is not properly understood, but the most plausible of previous

explanations is that a high calcium intake results in increased excretion of iodine, thus causing a deficiency of this essential element. This may well be the case, and it is possible that the supplement of sodium iodide, large though it was, was yet too small, and that if it had been greater a rise in haemoglobin concentration would have paralleled the histological changes in the thyroid gland. On the other hand, an alternative, or additional, explanation can be suggested which would also fit the facts disclosed by my findings: it may be that a high intake of calcium either blocks some stage in the synthesis of thyroxin or prevents its release from the thyroid gland. In the absence of fundamental understanding of the relationship between colloid formation and thyroxin production, the merits of this suggestion cannot be properly assessed.

The precise means by which thyroxin increases the level of circulating haemoglobin is another unsolved problem; but these studies have shown that desiccated thyroid was able to raise the haemoglobin level when iron was a limiting factor, so that thyroid hormone must in some way or other increase the efficiency of iron utilisation for haemoglobin synthesis. A connection between iron and thyroid hormone has also been established in a number of recently published papers, and the metabolic relationship between them deserves further examination. Indeed, there are important gaps in our understanding of all these matters, and the whole question of the part played by inorganic ions in the regulation of thyroid activity is worthy of closer attention than it has hitherto received.

Incidentally, the experiments reported here have shown that the Sherman B diet is far from perfect as a stock diet for breeding mice, although Sherman and his co-workers found it very satisfactory for their rats (see p. 119). In the first place, the diet seems to be deficient in iron, as shown by the fact that the haemoglobin level of the control animals on this diet was considerably increased by the administration of an iron supplement. The diet

also appears to be deficient in iodine, for the reason that thyroid hyperplasia was evident in the offspring of these control mice and was corrected by a supplement of sodium iodide. It must be emphasised, however, that these observations refer to the use of the diet for mice; when breeding rats were fed on this diet, neither they nor their offspring became anaemic, and certainly they proved to be much more resistant than mice to the deleterious effects of dietary calcium carbonate. The reason for this difference has not been studied, but it may be related to the stores of iron carried by each species. It has been pointed out at an earlier stage that there are differences between the two species both in the levels of their normal iron stores and in the rates at which these are built up. If calcium carbonate interferes with iron absorption from the gut, it might be the case that rats and mice are equally intolerant of calcium carbonate insofar as the absorption of iron is concerned, but that signs of frank iron deficiency, such as alterations in the blood picture, are not so readily induced in rats because of their greater iron reserves. The question might be settled by maintaining rats on iron-deficient diets over several generations, with the object of progressively reducing their iron stores, and noting whether or not calcium carbonate was more harmful under these conditions.

The studies reported here also showed that the effects of dietary calcium carbonate were not abolished by the addition to the diet of equivalent amounts of inorganic phosphate. In a few instances the phosphate appeared to moderate these effects to some extent, but on the other hand there was also evidence that additional phosphate actually hindered iron absorption or utilisation. On the whole, however, the haemoglobin concentration and the other erythrocytic attributes were not dependent to any important extent on the calcium : phosphorus ratio of the diet. This finding does not accord with the conclusions of some other investigators, but a review of published work on calcium-iron-phosphate

relationships shows that there is a distinct division of opinion concerning the effects of dietary calcium and phosphate salts, and especially the influence of the Ca : P ratio, on iron absorption and utilisation. The evidence for each point of view will now be discussed.

On the one hand, Kletzien (1935, 1938, 1940) observed that mice became anaemic when fed wholly on a commercial dog biscuit containing over 3½% of calcium, and he found experimentally that the assimilation of iron was inhibited when growing rats were fed a complete diet to which calcium carbonate had been added at a rate of 1% or 3%. The same result was obtained with several other salts of calcium, including the chloride, tribasic phosphate, citrate, tartrate, acetate and malate. The carbonates of beryllium, strontium and barium also inhibited iron assimilation, but those of sodium and potassium had the reverse effect. Kletzien therefore concluded that elements "fall according to their ability to facilitate or inhibit iron assimilation into either group 1 or 2 of the periodic system". He was unable to find any evidence that dietary phosphate inhibited iron assimilation, and, indeed, stated that a low dietary Ca : P ratio improved it appreciably. Kletzien and Kingdon (1936) also reported that when breeding female rats were fed a diet high in calcium, successive litters showed a progressive decrease in their carcase iron, and that this was most marked when the iron intake of the mother was low.

Shelling and Josephs (1934) also believed that a high Ca : P ratio adversely affected iron retention and blood haemoglobin although they admitted that their diets were not strictly comparable and their experiments not properly controlled. They reported further that the addition of vitamin D to high-calcium diets did not affect iron retention, although it seemed to improve it when the diets fed were low in calcium but high in phosphate - a finding subsequently confirmed by Fuhr and Steenbock (1943a, b). A more satisfactory experiment was reported by Anderson, McDonough and Elvehjem (1939-40). By means of supplements of calcium

carbonate and sodium di-hydrogen phosphate they varied the calcium and phosphate intakes of rats on a dry ration, and found that high levels of calcium carbonate retarded iron utilisation while the phosphate supplement had the opposite effect; iron retention was best with a low Ca : P ratio (0.45) and poorest with a high one (7.65).

Further evidence of a similar kind has come from Freeman and Ivy (1942) who found that the administration of calcium carbonate to anaemic rats reduced their iron retention, even when additional iron was fed. Fuhr and Steenbock (1943a) also noted that iron storage and blood haemoglobin were reduced by a high level of dietary calcium, and reported (1943c) that anaemia did not supervene when rickets was induced solely by a low intake of either calcium or phosphate. Robscheit-Robbins and Whipple (1930b) observed a drop in the haemoglobin level of rats during and after the feeding of calcium phosphate, and Nordfeldt (1939) showed that while mature female rats on a diet of cows' milk were already in negative balance, the addition of calcium lactate to the milk rendered the balance even more negative. Tompsett (1940) is another who has suggested that a high calcium intake may interfere with iron absorption, and De and Basu (1949) came to the same conclusion following short-period balance experiments on human subjects. A recent report from Pett (1952) shows that children whose bread was fortified with bone meal as well as iron and vitamins were more anaemic than children whose bread was totally unsupplemented, and the author suggested that iron absorption may have been interfered with by the added bone meal (the Ca : P ratio of bone meal is about 2.2 : 1). Rothlin and Schalch (1946) also have suggested that calcium and iron are antagonistic, from their finding that the intravenous injection of various salts of calcium protected white mice against later injections of toxic doses of iron salts.

On the other hand, the view that iron absorption is increased by a high level of dietary calcium or a high Ca : P ratio has gained wide acceptance. It

seems to date from iron balance experiments by von Wendt (1905) and Sherman (1907), who concluded that diets high in calcium increased iron retention in man. These experiments were carried out by comparatively crude methods, and have also been criticised by Davidson and Leitch (1933-34) and Widdowson and McCance (1936) on the grounds that experiments of this nature, lasting only 3 to 5 days, take no account of the influence of iron stores.

However, there is considerable evidence of more recent date to support their conclusions. Amongst the most formidable is that of the Yale group of workers. In a series of papers (Smith and Swanson, 1929; Swanson, Schultz and Smith, 1929-30; Smith and Schultz, 1930; Swanson and Smith, 1932a, b, 1934) they showed that normal rats fed on a diet containing only 0.5% of ash developed blood changes characterised by polycythaemia, low haemoglobin concentration and low packed cell volume; the mean cell haemoglobin and mean cell volume were consequently also both low, as was the mean cell diameter, but the mean cell haemoglobin concentration remained remarkably constant. "The picture.... is that of an infantile blood with a relatively enormous over-production of small, anemic cells" (Smith and Swanson, 1929), and the blood changes, which were progressive over a 90-day experimental period, were accompanied by clinical signs of ill-health. Control rats receiving a complete salt mixture (Osborne and Mendel, 1917) were unaffected, and affected rats could be cured by the administration of this mineral supplement. Orten and Smith (1934a, b) showed that whereas normal rats at 70 days of age had a reticulocyte count equal to 1-3% of the total red cells, the 'low salt'-fed rats developed a reticulocytosis equivalent to 14-28% of the erythrocytes; but this reticulocytosis developed only after the anaemia had become evident, and did not account for the initial polycythaemia. It should be noted that this whole syndrome is in many respect suggestive of the earlier stages of iron-deficiency anaemia. The basal diet consisted of casein, starch and lard, and when the

casein was replaced by edestin (a protein with a much lower phosphorus content than casein) the polycythaemia did not supervene and the other changes were much less marked (Swanson and Smith, 1932c; Swanson, Timson and Frazier, 1935). Because of this, and because most of the ash in the original diet was phosphate, Orten, Smith and Mendel (1936) attempted to counter its effects by adding pure calcium carbonate to the original 'low salt' diet, and found it immediately effective in preventing or curing the polycythaemic anaemia. An iron salt gave similar though less constant results.

From this it would seem that, with a diet low in calcium, even small quantities of phosphate can interfere with iron assimilation, while calcium carbonate has the effect of making iron available. In this connection it is noteworthy that a remarkably close parallel has been observed between the amounts of calcium and of iron in the livers of human foetuses and infants (Ramage, Sheldon and Sheldon, 1933; Sheldon, 1934). From their data, Ramage et al. suggested that the relationship might be one-sided, and that 'without the presence in the tissues of an adequate amount of calcium, the foetus or child is unable to store iron'; and consequently they also suggested that an adequate supply of calcium is necessary for the optimum absorption of iron.

A few years later, Day and Stein (1938) were able to reproduce polycythaemia and anaemia by feeding rats on the ration used by Orten et al. (1936), and, like them, succeeded in rectifying the blood changes by the addition to the diet of calcium carbonate (equivalent to 50 mg. of Ca per rat daily). They also found that 1.4 mg. of Fe as ferric chloride partially prevented the blood changes, and that 6 mg. completely prevented them; they further found that a supplement of beryllium carbonate containing 0.9 mg. of Be was similarly effective in the absence of iron or calcium. On the other hand, 6 mg. of Fe as ferric phosphate proved ineffective, as did 50 mg. of Ca as dicalcium phosphate. In a second series of experiments involving rats fed on a basal synthetic ration of similar

type, but fortified with vitamins and also a salt mixture (which did not, however, contain any calcium, phosphorus or iron), they were able to vary the intakes of these three elements by means of supplements of calcium carbonate, dihydrogen ammonium phosphate and ferric chloride. Whenever the Ca : P ratio was narrow, they found that polycythaemia and anaemia developed whether the absolute quantities of calcium and phosphorus were high or low, but restoration of the Ca : P ratio to normal prevented the blood changes provided that the diet contained iron. When normal quantities of the calcium and phosphate supplements were given without additional iron, similar blood changes became evident, thus showing that they were caused by iron deficiency. The authors therefore claimed that the effect of calcium on iron assimilation resides only in its ability to bind phosphate, thus permitting dietary iron to be absorbed instead of being excreted - presumably as an iron phosphate. But they also pointed out that this effect of calcium is neither invariable or specific, the essential property being an ability to bind phosphate.

Anderson et al. (1939-40) challenged these conclusions, which did not accord with their own results, and made the surprising - and unsupported - statement that 'from a strictly chemical point of view there is no reason to suspect that high levels of phosphorus should prevent iron assimilation'. This conjecture seems not only biased but rash, for the explanation put forward by Day and Stein is quite plausible. Moreover, it received strong support from the fact that a negative phosphorus balance, and even a marked degree of phosphorus deficiency, have frequently been induced in animals by adding to the diet an excess of a soluble salt of iron (Waltner, 1927; Cox, Dodds, Wigman and Murphy, 1931; Brock and Diamond, 1934; Deobald and Elvehjem, 1935; Elvehjem, Siemers and Mendenhall, 1935; Rehm and Winters, 1940). This syndrome is well-recognised, and has come to be known as 'iron rickets'. While Barer and Fowler (1940) found, in a short-period balance experiment on human subjects, that the administration of

iron did not affect calcium or phosphorus retentions, on the other hand Liu and Chu (1943) have found dietary iron useful in relieving the hyperphosphataemia associated with renal osteodystrophy in man, and Brock (1937b) showed that large oral doses of iron increased the ash content of rats' faeces, much of this increase being phosphate. Presumably the action of iron in all these cases depends upon the formation of an insoluble iron phosphate within the alimentary canal, and there should be no reason to suppose that a similar reaction cannot take place when the diet is high in phosphate and low in iron: indeed the situation is analogous to that obtaining in rickets, in which, when the diet is high in phosphate and low in calcium, a deficiency of calcium results from a comparable state of affairs. In any case, it has been shown (Brock and Taylor, 1934) that phosphates may inhibit iron absorption even when no precipitate is formed, since the dialysis of iron salts across cellophane membranes is greatly decreased in the presence of neutral or alkaline phosphates.

The view that high levels of phosphate will interfere with iron absorption received support from McCance, Edgecombe and Widdowson (1943), who found that iron absorption was decreased when phytates or phosphates were fed, but that the administration of calcium diminished this effect. Further evidence that a narrow Ca : P ratio can cause anaemia was provided by Happ (1922) who noted that rats fed on low calcium-high phosphorus rachitogenic diets often became anaemic as well as rachitic, but that rats on high calcium-low phosphorus rachitogenic diets showed no such tendency. Indeed, when Hegsted, Finch and Kinney (1948, 1949) and Kinney, Hegsted and Finch (1949) fed rats on a low phosphate diet of corn grits and lard with an iron supplement, they noted that iron absorption was so high as to cause haemosiderosis; the addition to the diet of phosphate reduced iron retention considerably, but a supplement of calcium carbonate had no effect. These workers postulated that the 'mucosal block' to iron absorption can break down either in the absence of an adequate supply of

phosphate or when iron intake is excessive. Incidentally, it has been suggested (Anonymous, 1950a) that the haemosiderosis commonly seen in ill-nourished Bantu pellagrins (Gillman, Mandelstam and Gillman, 1945) may have a similar aetiology. Although Walker and Arvidsson (1950) and Walker (1951) later pointed out that the diet of the Bantu is normally high in phosphate, they also showed that iron intake may be very high; it therefore seems possible that a high dietary Fe : P ratio may indeed be implicated in this condition.

From what has been said, it is clear that a sharp conflict of opinion exists concerning the influence exerted by dietary calcium and phosphorus on iron assimilation, and certainly the literature contains considerable evidence to support both points of view. Hynes (1948) has pointed out that an excess of calcium can both inhibit and favour the absorption of iron, and Cartwright (1947), following a brief review of the subject, concluded that there is disagreement as to whether a high or a low Ca : P ratio is more favourable for iron utilisation. These statements no doubt represent the present situation fairly, but nevertheless the opinion that iron assimilation is favoured by a high Ca : P ratio seems to have gained the wider acceptance. For instance, McLester (1927-49) states that calcium "has important iron-sparing qualities" and that "with abundant calcium, the organism can maintain equilibrium on a smaller supply of iron". Davidson, Fullerton, Howie, Croll, Orr and Godden (1933) stated that "the iron-sparing qualities of calcium are of practical importance: it is definitely known that a normal blood level can be maintained on a diet rich in calcium and moderate in iron, but that anaemia will occur on the same intake if the calcium is reduced". Sheldon (1934) follows the same line of thought when saying that it has been known for some time that "in some way calcium conserves iron in metabolism". Sherman (1932) also refers to this conservation of iron by dietary calcium, while, in a review on mineral metabolism, McCance and Widdowson (1944a) state that the absorption of iron is liable to be

interfered with by precipitation in the intestine, and that phosphates are of practical importance in this respect. Further, in a recent leading article on the absorption of iron (Anonymous, 1951) there appears this sentence: "The interference (with iron absorption) by phosphate is generally accepted by authorities on mineral metabolism". Piney (1939) states that "calcium in adequate doses reduced the amount of iron needed for cure of a deficiency anaemia", and Kugelmass (1940) states "abundant supply of calcium .... in the diet favors a positive iron balance on smaller intake". Bacharach, Cuthbertson and Thornton (1949) apparently believed this to be the case when they administered calcium as well as iron in treating a natural outbreak of iron-deficiency in stock rats, notwithstanding that the calcium intake was already high. Parsons, Hickman and Finch (1937) and Parsons (1938), whose minds evidently also ran along these lines, observed a number of similarities in the metabolism of iron and calcium, and concluded that cases of iron-deficiency anaemia might benefit from treatment with calcium salts; however, they obtained no response to repeated medication with calcium chloride. Nevertheless, Whitby and Britton (1946) state that following excessive blood loss the administration of calcium may be "of value in bringing about a more rapid replacement of the consequent iron deficiency."

The results of my own work support the first, less widely-held view, namely that calcium salts interfere with iron absorption. At the same time, the iron-phosphate interactions which were observed point to the likelihood that the second view - that phosphates interfere with iron absorption - is also a correct one. With so much directly conflicting evidence, it is impossible to do other than conclude that both views may be right and that the actions of dietary calcium and phosphorus upon iron utilisation must be governed by some other important factor, qualitative or quantitative, which has not yet been defined.

It will not be possible to identify such a factor, and so resolve the controversy, until more is learnt of the modus operandi of calcium carbonate in these experiments. No conclusions can be drawn from the present studies on the mechanism at work, but this is clearly the next question inviting investigation and at this stage it will be appropriate to discuss in broad outline how the problem could be attacked.

In the first place, it must be determined whether the calcium or the carbonate ion is the radicle responsible. A third possibility, of course, is that the effect is exercised by the undissociated calcium carbonate molecule rather than by one of its constituent ions. The answer to this question should be discoverable by supplementing the basal Sherman B diet with other salts of calcium and with other salts of carbonic acid, and comparing their effects with those given by calcium carbonate.

If it were to be found that the calcium ion is the factor responsible, its site of action would next have to be discovered; in the first place this would mean determining whether it acts before or after absorption from the alimentary tract. The finding in the present work that the serum calcium concentration remains unaltered is not particularly helpful; it cannot indicate whether calcium absorption was excessive or not, for serum calcium is maintained at a remarkably constant level under widely varying circumstances. Even when absorption of calcium is increased, unless it be very excessive it is not followed by hypercalcaemia, calcium being stored or excreted as fast as it is absorbed. However, there is no reason to think that absorption of calcium should have been interfered with in this case, as the diet presumably contained adequate amounts of vitamin D owing to its very high content of dried milk; and indeed it appears that unusually large quantities of calcium were, in fact, absorbed, because hypothyroidism was intensified when a high calcium carbonate diet was fed, and, as we have seen, calcium is goitrogenic only after it has

been absorbed.

With the serum calcium level normal, however, it is difficult to imagine in what way absorbed calcium can interfere with the utilisation of iron for haemoglobin synthesis. It is just conceivable, of course, that excessive excretion of calcium might have some effect on the retention of dietary iron, but from what is known of the mechanism of iron absorption and metabolism this appears to be a most unlikely proposition. The point could easily be settled, however, by administering calcium by injection instead of by mouth, and observing whether or not similar consequences ensued.

On the other hand, calcium ions may interfere with the absorption of iron before they are themselves absorbed. However, it does not seem likely that they could do so by means of a simple chemical reaction: calcium does not form insoluble or unionisable double salts with iron (except in the case of an acid such as phytic acid, where the fact is immaterial because the simple iron salt is fully as insoluble, if not more so, than is the double salt); nor is calcium recognised to form any other insoluble complexes with iron. Another possibility, however, is that in forming an insoluble salt with, for example, an orthophosphate anion, calcium may bring iron out of solution as a result of co-precipitation. This suggestion seems well worth further consideration; probably the initial stages of its investigation would be best conducted by means of <u>in vitro</u> experiments.

It may be, however, that the carbonate radicle is the operative factor. As carbonic acid is such a weak acid, calcium carbonate is an alkaline salt, and as such would be expected to raise the pH of the stomach and intestinal contents; indeed, it is a well-recognised antacid. Iron salts are not only insoluble in alkaline solution, but are readily precipitated, as hydroxides, well on the acid side of neutrality (there are some exceptions, such as the citrate and tartrate, which form complex metallic anions). It is this fact

that accounts for the well-recognised association of iron-deficiency anaemia in the human subject with gastric anacidity, and it also explains why heavy liming of soils may render iron unavailable to plants. Prima facie, it seems not unlikely that the alkaline nature of the calcium carbonate supplement could result in precipitation of the soluble iron compounds in the diet.

A further possibility must not be overlooked, however. Calcium carbonate possesses a strong adsorptive action, and indeed because of this property it is frequently used as a purifying agent in certain industrial processes. It is therefore possible that the insoluble calcium carbonate molecule removes soluble iron from the diet by a process of adsorption; this possibility is another which seems to deserve further examination, and again in vitro methods might be useful at first.

The antagonism between dietary calcium carbonate and dietary cobalt also calls for investigation, and it seems quite likely that this phenomenon could be governed by the same mechanism as that involved in the interaction between calcium carbonate and iron. In this connection it is worth remembering that the elements iron and cobalt are closely allied in many of their properties, and occupy adjoining positions in Group VIII of the Periodic Table.

The findings reported in this thesis thus open the way to a further series of investigations. Before these are undertaken, it might be well to consider whether any improvements can be made in the general technique employed. The greatest single difficulty encountered in the experiments described here was the fact that when iron deficiency was severe the survival rate of the weanlings was very poor. This often resulted in entire litters perishing before reaching weaning age, and so constituting a number of missing observations. Although in many cases statistical analysis of the data was still possible, the accuracy of the estimated effects was often gravely reduced, and even large differences could not be described as significant. In future studies, this difficulty might

be avoided if it were found possible to alter the procedure so as to measure the extent of iron deprivation on animals other than weanlings. Young adult animals might be employed, but it has to be remembered that iron, once absorbed, is not excreted from the body except in minute quantities and is used over and over again for haemoglobin formation, so that the mere fact of a low intake or absorption of iron is not of itself sufficient to induce signs of deficiency unless at the same time the demand for iron is increased. We have already demonstrated the truth of this point, as the young adult males used for mating were generally little affected by the supplement. An increase in the demand for iron can be brought about in a number of ways. First there is the method employed so far in these studies - the stress of pregnancy and lactation; this drains iron from mother to young, and if there is insufficient iron to meet the needs of both the deficiency becomes manifest in both - in the form of anaemia. Another method which has been much used by other investigators is single or repeated haemorrhage, which has the effect of exhausting the reserves of iron and so rendering the animal anaemic and thus capable of showing a response to absorbed iron. As a modification of this, the same end could probably be achieved simply by breeding mice over several generations either on iron-deficient diets or - in view of the findings in this thesis - on diets containing high levels of calcium carbonate. Finally, use might be made of the fact that the demand of young animals for iron continues to increase so long as they are growing; not only does the blood volume expand, but every other iron-containing tissue does so as well (and these have priority over the blood when a deficiency arises). Thus by using young growing animals it might be possible to infer from changes in the blood haemoglobin concentration whether dietary iron was being utilised to the full. Although this technique has been much used in the past in experiments on iron metabolism in rats, when used in young mice it suffers from the great disadvantage that - simply on account of their

very small size - repeated samples of blood cannot be obtained from the living animals. Consequently it would not be possible to determine the initial haemoglobin concentration of the experimental animals; but this difficulty might be largely overcome by the use of littermates, which generally have very similar levels of iron stores and so would be expected to have similar initial haemoglobin levels. If this method were to prove unsuccessful, recourse might have to be made to carcase analyses, but as these are very time-consuming and require special precautions to avoid contamination from atmospheric dust particles and other sources of error, their use should be avoided if possible.

In conclusion, the possible practical importance of the findings reported in this thesis may be briefly considered, although it will not be possible to assess their full significance until the mechanism at work has been elucidated.

Domestic animals, particularly ruminants, are not commonly subject to iron-deficiency anaemia, and their reserves of iron are usually plentiful. Under natural conditions of management, the diet should contain a sufficiency of iron, while further opportunities to augment the supply of this element are provided by adventitious intakes from the soil during grazing, and from cleaning or licking of the coat. In some parts of Florida (and possibly elsewhere) the soil is deficient in iron, and under these conditions iron-deficiency anaemia has been recorded in cattle; but such conditions are very exceptional, and, while iron deficiency might follow a severe haemorrhage, or even a heavy loss of blood such as occurs in the haemoglobinuria associated with some forms of piroplasmosis, it is nevertheless safe to say that, in general, iron deficiency is not commonly encountered in animals maintained under natural conditions. Under these circumstances, therefore, although the present practice of administering to livestock large amounts of mineral supplements - many containing a high proportion of calcium, often in the form of the carbonate - may limit the availability of dietary iron, it seems hardly likely to involve much risk of

inducing a frank deficiency.

When conditions of management are more artificial, however, the danger of an iron deficiency is much more real, and it seems to be particularly serious in the pig. It is well recognised, of course, that if sucking pigs are reared indoors and not supplied with iron in some form or other, they develop iron-deficiency anaemia within a few days, their iron stores at birth being insufficient to meet the demands of rapid growth. If the rations of a sow were heavily supplemented with calcium carbonate she might be unable to replenish her body stores of iron, which, if she were carrying a large litter, would be heavily depleted during pregnancy; consequently the already inadequate birth stores in the piglets would be further reduced. Similarly, if growing pigs under modern intensive systems of fattening are denied access to soil and fresh food, it seems quite possible that their iron stores may become depleted, and anaemia develop, if further supplies of iron are not provided. The danger of deficiency would be further increased if the only food available were supplemented with mineral mixtures containing large quantities of calcium carbonate.

While pigs are probably the only domestic mammals likely to be affected in this way, the case of poultry kept in batteries is also worth considering. Under this system of management, pullets are not only still growing when deprived of natural sources of iron on the ground, but very shortly the additional stress of egg-laying is superimposed upon that of growth. Unless their reserves of iron are very large, or some additional source of the metal is provided, it seems possible that here again iron deficiency may be a not unlikely consequence, particularly as the calcium intake has to be high when the birds are in lay.

It is not suggested that in any of the circumstances just described iron deficiency is recognised as an important problem. Even under artificial conditions, it is quite possible either that the iron stores of pigs and poultry

are adequate to meet all demands, or that other sources of iron are available to them - for example, rusty metal fittings. It is also possible that in these species of animals calcium carbonate is less harmful to the absorption or utilisation of iron than it is in mice. On the other hand, anaemia may frequently exist without being recognised; and as even mild degrees of anaemia can be detrimental to health, it is therefore suggested that the point is at least worth attention.

It is with man, however, that the interaction between dietary calcium carbonate and iron may be of most practical importance. Iron deficiency is a common condition in the human subject, particularly in growing children and in women. As has been indicated earlier, it is often recommended that calcium be administered together with iron to facilitate the absorption of the latter, the rationale of the advice being that calcium may prevent the precipitation of iron by phosphates and phytates. In the light of my findings, however, it is questionable whether this procedure may not be harmful rather than helpful. Species differences may exist, of course, and, as has already been pointed out, the findings of some others are not in agreement with mine, but nevertheless the question appears to merit much fuller examination.

Evidence has also been obtained in my studies that calcium carbonate in the diet interferes with the absorption or utilisation of cobalt, and also that there is an interplay between dietary calcium carbonate, iodine and the thyroid gland; further research may show that these points, too, have their practical applications as well as academic interest.

The real importance of the subject, however, may not yet be apparent, and there may well be several other examples of dietary antagonism awaiting discovery. The whole question of interactions between mineral constituents of the diet is far from being well understood, and in my opinion it is one that deserves the fullest investigation.

B. RÉSUMÉ

1. The starting point of this work was the observation that, when breeding mice are fed on a slight modification of the B diet of Sherman, the addition to the diet of calcium carbonate at the rate of 2 per cent. results in a serious impairment of their reproduction performance, especially their rearing performance. Following upon this finding, I studied the pathology of the dams and their litters in a preliminary experiment.

2. At weaning (21 days old), the young mice were ill-developed. The chief lesions observed in these animals were mild general oedema, increased stores of depot fat, pale mottled livers, enlarged hearts and atrophied thymus glands.
On subsequent histological examination, the livers showed fatty degeneration, sometimes of an extreme nature, and the heart muscle was oedematous; there was accidental involution of the thymus glands, a normoblastic reaction in the bone marrows, and in some cases early degenerative changes in other organs. Confirmation of all these lesions were obtained in later experiments.

3. As these changes were all suggestive of anaemia, a haematological examination was carried out on some animals from the preliminary experiment. This showed that both the adult and young mice from the diet-groups receiving calcium carbonate were anaemic compared with the control animals from the unsupplemented diet-groups, and that these in turn were anaemic when compared with comparable animals fed on stock diet; some evidence was obtained that in both instances the anaemia was microcytic and hypochromic in type. The weanlings from the supplemented diet-groups were also hyperlipaemic.

4. After consideration of the principles of normal and abnormal erythropoiesis, it was concluded that - although several other factors could not be excluded - the likeliest cause of the anaemia was an induced deficiency of iron. It was then found by experiment that the addition of a preparation of ferric citrate to the supplemented diet largely abolished the deleterious effects of the supplement. The blood pictures of the control animals were also improved by the addition of iron to the unsupplemented diet.

5. The iron supplement also countered the effects of dietary calcium carbonate on the weights of the hearts and thymus glands, and it improved the oedema in the heart muscle. It probably also effected a real improvement in the rearing performance.

6. Confirmation was obtained that the anaemia induced by calcium carbonate was hypochromic in both dams and litters, although it was more markedly so in the young animals; microcytosis, however, was evident only in the dams. After discussion, it was concluded that these differences could be ascribed largely to differences in the degree of the anaemia, which in the young animals was more severe and was accompanied by a flooding of the peripheral blood with large immature erythroid precursors. It was also noted that second litters were less anaemic than first litters, and it was suggested that the animals may have developed a toleration to the effects of the calcium carbonate supplement.

7. It was further observed that the addition to the diet of sodium di-hydrogen phosphate, in sufficient amount to restore the Ca : P ratio of the diet to its original level, failed to offset any of the effects of dietary calcium carbonate, although with the dams and second litters a minor improvement was seen in the haemoglobin concentration. In some instances, however, the

phosphate supplement appeared to diminish the beneficial effects of iron. It was concluded that the action of calcium carbonate does not depend upon alteration of the Ca : P ratio of the diet.

8. A close correlation was observed in weanlings between the heart weight : body weight ratio and the blood haemoglobin concentration, the relationship between the logarithms of these values being inverse and linear.

9. Although the effects of the iron supplement were everywhere in the opposite direction to those of the calcium carbonate supplement, it was pointed out that this alone was insufficient evidence for concluding that calcium carbonate interferes, even partially, with iron absorption or utilisation. Indeed, the iron supplement also benefited the control animals to a very similar extent, and no significant interaction was evident between iron and calcium carbonate. While these results might suggest, in fact, that calcium carbonate must produce its action in some other way than by interfering with iron, it was also pointed out that the response to iron medication was limited in the controls by the fact that their initial haemoglobin levels were much nearer the physiological optimum than were those of the animals receiving the calcium carbonate supplement, and that the magnitude of the interaction could therefore be taken to represent a balance between the limiting action of this 'room for improvement principle' in the control group and the limiting action of the calcium carbonate supplement in the supplemented group. It was concluded, therefore, that although the pathology of the affected animals suggested that calcium carbonate induces an iron deficiency, this hypothesis had not been proved; at the same time, however, no evidence had been obtained which was at variance with it.

0. In later experiments, more conclusive evidence was obtained that the anaemia

caused by dietary calcium carbonate was, in fact, mainly the consequence of an induced iron deficiency. In the first place, it was shown that the interaction on haemoglobin concentration between iron and calcium carbonate was much more evident when there was greater 'room for improvement', and in the second place it was shown that when the diet was very rich in iron calcium carbonate was relatively ineffective unless given in very large amount.

11. At the same time, certain other factors could not be excluded as additional causes. Although many causes of anaemia are known, consideration of their modes of action and of the circumstances reduced the number of factors which could have played a part in this case to five, namely, deficiencies of iron, copper, cobalt or pyridoxin, and hypothyroidism. Consideration of the known causes of the other pathological features observed in the mice from the supplemented groups indicated that all could have arisen secondarily to the anaemia. At the same time, a number of other factors could not be excluded as alternative or additional causes. The fatty livers might have resulted from diabetes mellitus, hypothyroidism or deficiency of a lipotropic agent such as choline or inositol; the hyperlipaemia might have been caused by diabetes mellitus or hypothyroidism; the impairment in reproduction efficiency might have been associated with deficiency of manganese, pyridoxin or inositol; and the thymus atrophy might also have been due to pyridoxin deficiency.

12. In view of these conclusions, further experiments were conducted to test the effects of supplementing the diet with copper, cobalt, manganese, desiccated thyroid gland, pyridoxin, inositol and choline. Cobalt and desiccated thyroid both increased the blood haemoglobin level, but none of the supplements significantly affected either the rearing performance or

any of the other abnormalities. It was also shown that diabetes mellitus was not responsible for any part of the syndrome, as the pancreatic cell-islets were not affected in animals fed on diets containing a high level of calcium carbonate. As on several occasions these abnormalities were found to be closely correlated with the severity of the anaemia, there can therefore be little doubt that all were secondary to this feature.

13. Cobalt is frequently a contaminant of iron preparations, and thus part of the effect produced by the ferric citrate preparation, which was not specially purified, may have been due to this cause. On the other hand, the purest available preparation of iron also raised the blood haemoglobin level, so that if an induced deficiency of cobalt played any part in the causation of the anaemia associated with dietary calcium carbonate (and this was shown to be very doubtful) it is likely that it was only a subsidiary and not the main cause. Nevertheless, it was noted that the effects of supplementary cobalt as well as iron were hindered by the presence of calcium carbonate in the diet, and this observation, especially if it can be confirmed in ruminants, is one that may be of practical importance.

14. While some degree of hyperplastic goitre was evident in weanlings from the control diet-groups, calcium carbonate was observed to cause extreme hyperplasia of the thyroid glands, indicating that the animals were in a severely hypothyroid state. It was also notable that desiccated thyroid was particularly effective in raising the blood haemoglobin concentration when the diet contained calcium carbonate. These results suggested that the anaemia may have been due in part to hypothyroidism. The goitrogenic action of calcium salts is not understood, but it has been suggested previously that it may act by inducing a deficiency of iodide. The addition

to the diet of a supplement of sodium iodide reduced the hyperplasia in the thyroid gland, but did not relieve the anaemia, although in the absence of calcium carbonate it did both. Consequently, it was suggested - although no definite conclusions could be drawn - that a high intake of calcium may interfere with the synthesis or release of thyroxin.

15. Experiments with several strains of mice showed that they did not differ in their susceptibility to dietary calcium carbonate; on the other hand, rats proved to be much more resistant. Although the reason for this difference is uncertain, it may be related to differing levels of iron reserves in the two species.

16. Estimations of serum calcium and inorganic phosphate levels showed that they were unaffected by a supplement of calcium carbonate in the diet.

17. The conclusion that calcium carbonate diminished iron absorption or utilisation, and that the latter is not influenced to any important extent by the Ca : P ratio of the diet, is at variance with the findings of several other workers. However, published opinion falls into two distinct groups on this point, one of which considers that a high Ca : P ratio is inimical to iron absorption, while the other believes that a high level of dietary calcium, by preventing the formation of insoluble iron phosphates, is valuable in assisting the absorption of iron. Of these two opinions, the latter is more widely held, but my results do not accord with it in any way. The evidence in its favour is so strong, however, that it was concluded that the effects of dietary calcium and phosphate upon iron utilisation must be governed by some other factor, qualitative or quantitative, which has not yet been defined.

18. The opinion was expressed that the controversy will not be resolved until

more has been learnt of the modus operandi of dietary calcium carbonate, and suggestions were put forward in regard to ways in which the problem might be attacked. Owing to difficulties met with in my experiments, a new technique for conducting such experiments may be advisable, and it was suggested that use might be made of the fact that the demand of young animals for iron continues to increase so long as they are growing.

19. Finally, the possible practical significance of the findings was briefly considered. Apart from the relationships between dietary calcium carbonate and cobalt, and calcium carbonate and the thyroid gland, the interaction between calcium carbonate and iron may have an important bearing on nutritional practices; the species which may be concerned include not only pigs and poultry, but also man. The opinion was expressed that the whole question of interactions between mineral constituents of the diet is one that deserves much fuller investigation.

APPENDIX I

TECHNICAL APPENDIX

TECHNICAL APPENDIX

In most of the experiments described in this thesis, the methods employed followed the same general pattern. It will be convenient to discuss these principles here, and to note any exceptional or special features when describing the particular experiment to which they refer.

A. DESIGN OF EXPERIMENTS AND ANALYSIS OF EXPERIMENTAL DATA

In experiments in which only a single variable is under test the design presents no difficulty, equal numbers of littermate animals being allocated to each of two treatments - one experimental, the other control. However, when it is desired to examine the effects of more than one variable, whether these be independent or interacting, the use of an experiment with a factorial design may be advantageous in that it enables more information to be obtained from a given number of animals. The advice and ready co-operation of Mr. A. W. Boyne, of the Statistics Department, The Rowett Research Institute, and of Mr. M. H. Quenouille, lately of the Statistics Department, University of Aberdeen, made proper use of factorial designs possible.

Because of limitations on the number and classes of animal available, or for other practical reasons, the ideal design proposed could not always be utilised, and compromises had sometimes to be effected by arranging the design so that maximum information would be given on certain main points, but only less definite answers could be obtained to other questions.

The greatest disadvantage of factorial designs for this type of work is the fact that a number of missing observations must be expected. However, when an experiment was complete the data were submitted to Mr. Boyne or Mr. Quenouille for statistical analysis, and in most cases the statistician was able to conduct

an accurate analysis after estimating any missing values. If these were very numerous, of course, the number of degrees of freedom thereby lost seriously prejudiced the likelihood of reaching a significant conclusion. The significance of differences (i.e. the probability of their being real, and not due to chance) was estimated by the t test (Fisher, 1948). While no level of significance can imply absolute certainty, for most purposes significance at the 5% level was accepted as being meaningful. Sometimes, however, circumstances warranted the raising or lowering of this arbitrary standard.

In allocating animals to treatments, the two chief variables which could be controlled were (1) the littermate factor and (2) the arrangement of the animals comprising each litter. The littermate or 'inheritance' factor is particularly important in experiments involving iron-deficiency, because so much depends upon the pre-natal stores of iron laid down in the foetus; it very soon became apparent that littermates behaved much more similarly than did non-littermates. Consequently, littermates (usually four in number) were always employed, and usually they were stratified through the design. Where only four treatments were being compared, one member of each litter was allocated to each treatment, so that litter-mate effects would cancel out in the treatment means. But with more than four treatments, the littermates had to be allocated in such a way that litter effects could be isolated and eliminated before treatment means and their standard errors were calculated. The effect of the littermate factor was also an important consideration in the estimation of missing observations.

The second variable - the arrangement of animals within litters - was much less important. Differences between these animals were usually very small, and in most cases probably of no significance; if a litter appeared uneven it was not used. Nevertheless, since it was possible - and involved very little extra work - the individuals in each littermate quartet were either stratified by weight within the design or else distributed at random; for this

latter purpose a sheet of random numbers was employed, each animal having been previously designated No. 1, 2, 3 or 4 according to its position in descending order of weight.

B. ANIMALS

Mice. The mice used in most of the experiments were a Webster Swiss albino variety of Mus musculus from the Rowett Research Institute's stock colony. The history of this colony has been described (Sengupta and Howie, 1948-9); it is kept as a closed population without brother-sister mating, and its members are now reasonably uniform in weight, size, rate of growth, and many other biological characteristics. Experiments were also performed with three other strains of mice: one was the black C57 strain, another was a coloured hybrid strain, and the third a recently-developed 'silver' strain obtained from the M.R.C. Laboratory Animals Bureau, Hampstead, London.

To supply experimental animals for each experiment, breeding had to commence between 10 and 11 weeks before the animals were required; but preparations had to be made well before this to ensure that sufficient breeding females would be available. Plans for future work had therefore to be made several months before they could be executed.

As indicated in the previous section, the object was to breed littermate quartets of male and female mice. Experience with the colony had shown that to be reasonably certain of securing sufficient quartets, the number of breeding females required was approximately equal to the total number of quartets to be supplied. Moreover, if the litters were not to differ in age by more than 4 days, and to allow for the discarding of uneven or otherwise unsatisfactory litters, not only had all to be mated on the same day but the number of breeding females had to be increased by nearly 50 per cent. (Porter, G. Private communication). Thus if 64 mating pairs (i.e. 16 male and 16 female quartets,

total 128 mice) were required for an experiment, from 45 to 50 stock breeding females had to be mated on the same day to ensure the requisite numbers of suitable experimental animals.

The breeding females were fed on stock diet, which consisted of Rowett Institute Stock Cubes plus a daily supplement of fresh whole cows' milk. The cubes contained:

Wheat offal (bran)	17.7
Wheat, whole ground	17.7
Oats, Sussex ground	17.7
Maize, ground	8.8
Barley, ground	8.8
White fish meal	4.5
Meat-and-bone meal	8.8
Dried skim milk	14.0
Dried yeast	1.2
Sodium chloride	0.4
Cod liver oil	0.4
	100.0

Both cubes and milk were fed ad lib., and ample fresh water was supplied daily.

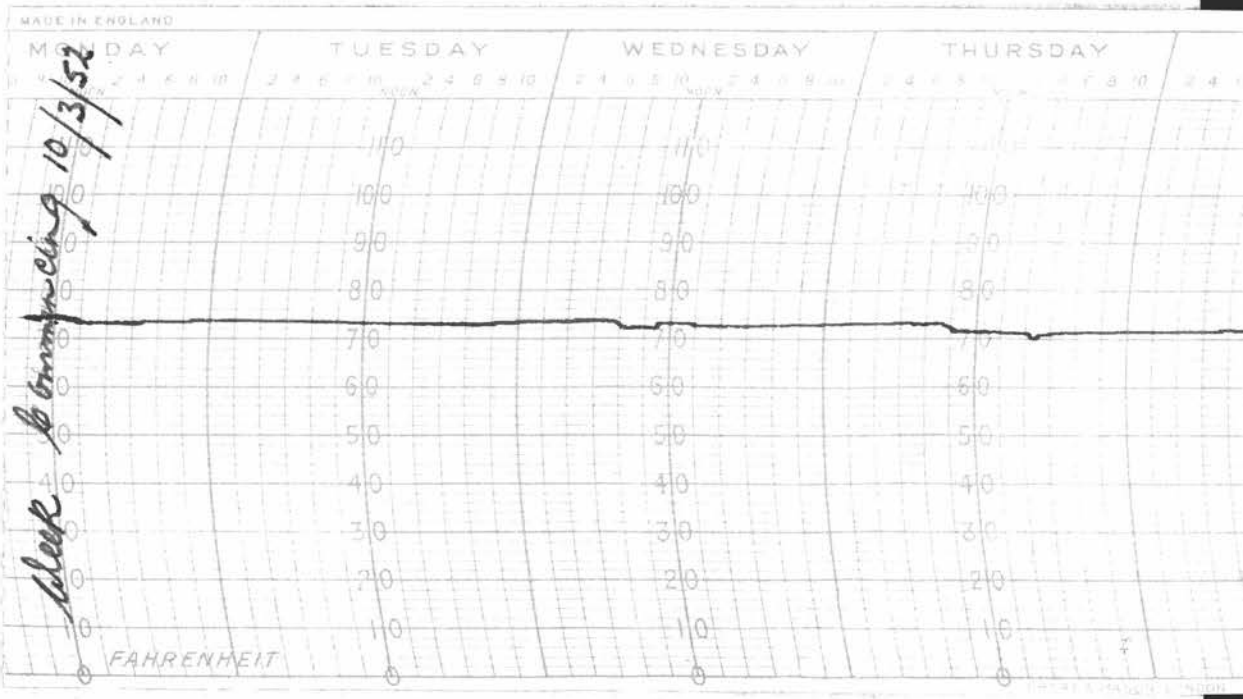
The room in which the animals were kept was heat-insulated and air-conditioned, the inflowing air being filtered and warmed. Thermostatic control of the temperature of the air inflow maintained the room-temperature very close to 72° F. for 24 hours a day in both summer and winter; temperature was checked by thermograph recordings (see Fig. 36).

The female mice were mated (usually 4 or 5 females per male) in galvanised iron-wire cages measuring 12 in. x 12 in. x 6 in., mesh ½ in., and transferred, when pregnant, to smaller (6 in. x 4 in. x 4 in.) but otherwise similar individual cages, where they were allowed to litter down. The cages rested on trays of sawdust, and wood wool was given for bedding and nesting purposes.

The litters resulting from these matings were weaned at 21 days, weighed, and separated into part-litters according to sex; part-litters of four or more

FIGURE 36

Thermograph recorded in Small-Animal House



were retained as experimental animals. These were reared on the stock diet described above until the age of seven or eight weeks, when each animal was weighed, allocated its place in the experimental design, and given its appropriate experimental diet. In some instances, feeding of the experimental diet commenced earlier than seven weeks, following the technique of Deuel, Meserve, Straub, Hendrick and Scheer (1947).

The system of continuous monogamous mating advocated by Bruce and Emmens (1948) was employed, with the modification that males were removed from the cage, not after a specified interval of time, but after the conception by their mates of a specified number of pregnancies (usually two). The mating age varied from seven to nine weeks, but was kept uniform within each experiment.

The cages used for the experimental mated pairs were similar to the small breeding cages described above. In later experiments, an improved cage measuring 12 in. x 5 in. x 5 in., mesh $\frac{1}{4}$ in., was employed. Care was taken to see that cages remained free from rust or other source of metallic contamination. They were distributed evenly around the room, supported by open shelves 12 in. apart, which permitted free circulation of air. A minimum clearance of 2 in. was maintained between each cage. The sawdust on which they rested was changed twice weekly, as was the wood wool used for bedding. Room temperature was maintained at about 72° F.

All animals were fed once daily except on Sundays; on Sundays no feed was given, but a double quantity was fed on Saturdays. The weight of food supplied and the weight of residual uneaten food were recorded daily, the quantity fed each day being 3 or 4 g. more than the quantity consumed during the previous 24 hours. This procedure prevented undue waste of food and yet ensured that each animal was offered sufficient. Feed was prepared daily, being mixed in the ratio of 3 g. of dry food to 1 ml. of distilled water, and then kneaded into a stiff dough. Water bottles were replenished daily with

distilled water; once a week they were sterilised by boiling.

Experimental diets were prepared in small batches, preferably not more than 2 or 3 kg. at a time, to ensure thorough mixing of their ingredients. The main bulk of the ingredients, such as wheat, dried milk and casein, were first mixed together in their proper proportions and spread out in a thin layer over a large plastic-covered sheet. The mineral salts - sodium chloride, calcium carbonate, etc. - were dried in an oven before being weighed out into a mortar and ground into a fine powder; when small quantities of supplementary substances were to be included, they were added to the salt mixture at this stage and the whole thoroughly compounded. Finally, the contents of the mortar were sprinkled evenly over the main bulk of the diet, and incorporated into it. The final mixing, which extended over 15 or 20 minutes, was accomplished either by hand or in a ball mill. In some instances where only traces of a soluble supplement were to be added, it was found more satisfactory to dissolve it, in proper concentration, in the distilled water used for making the dough.

Rats. The two experiments with rats (Rattus norvegicus) were done with animals from the Rowett Research Institute's stock colony of Hooded Listers. This colony has also been maintained as a closed population for several years, and its members are uniform in most respects. They are maintained on the stock diet described above. Because of the relatively small numbers involved in these experiments no special arrangements were necessary for breeding the experimental animals.

In these experiments, mating was commenced at thirteen weeks of age and was polygamous, one male being placed with four females in a galvanised iron-wire cage 12 in. x 6 in. x 6 in., mesh ½ in. When pregnant, females were transferred to individual cages of similar size and design. Room temperature

was maintained at 72° F. Arrangements for feeding were similar to those described above for mice.

C. COLLECTION OF MATERIAL

Litters were counted at birth and on every succeeding day until weaning, detailed records being kept of any deaths or other important events. Mice were allowed to rear as many of their young as possible, but, following the practice in the colony, litters of rats were reduced to a maximum of eight at three days old. Mice were weaned at 21 days of age; rats at 24 days. At that stage the young animals were sexed, and the total numbers and weights weaned were recorded separately for males and females.

The weanlings were then examined as soon as possible in the laboratory. In most experiments each litter was represented, where possible, by two males and two females; these were the two animals nearest to the median weight of each sex. In all the earlier experiments, anaesthesia was induced under a large inverted filter funnel in which was placed a small piece of absorbent cotton wool soaked in ether. The axillary vessels were then resected and samples of blood collected as required, the animal being forthwith destroyed by further exposure to ether vapour. Tissues for histological examination were then dissected out, and in some instances the weights of certain organs were recorded; the carcase was thereafter disposed of by incineration.

The dams were dealt with, in the same way, on the day that their final litter weaned. Sires were similarly treated when it became certain that their mates were carrying their final litter.

In place of anaesthesia, a simpler, quicker and equally humane method of preparing small animals for blood collection in non-survival experiments consists in stunning them by means of a single sharp blow on the back of the head. This method has the disadvantage that the blood clots very rapidly,

but it was found suitable when only a single operation, such as haemoglobin estimation, was to be performed on fresh blood. It was questionable, however, whether this procedure would lead to results comparable with those obtained following the use of ether anaesthesia. A number of reports in the literature have suggested that ether anaesthesia distorts the blood picture, largely by inducing haemonoconcentration; but there has been considerable controversy on the subject, and many workers have been unable to find appreciable alterations in the concentration of the cellular elements following ether administration.

Consideration of the literature (which is reviewed on p. 265) suggested to me that species differences might, in part, account for the conflicting nature of the reports. It therefore seemed worth while to compare the relative effects of ether and of stunning on weanling mice, especially since I had been unable to find any literature dealing with the effects of ether on the blood of this species. My investigation, which is described on p. 267, clearly showed that the haemoglobin concentration of mice following stunning is greater than that following ether anaesthesia, and it therefore followed that, in collecting blood samples from mice, ether anaesthesia and stunning could not be used interchangeably. Consequently, although in some later experiments the animals were stunned instead of anaesthetised, whatever the method employed it was retained throughout the whole experiment.

D. TECHNICAL METHODS

1. Haematological methods

ANTICOAGULANT

The use of an anticoagulant is unnecessary when the only procedures intended are estimations of haemoglobin content and numbers of erythrocytes and leucocytes, but it is essential if an estimation of packed cell volume is also

to be made. In Experiment 1 heparin was used as the anticoagulant. While this substance may be considered the 'natural' anticoagulant, Whitby and Britton (1950) state that its use is unsatisfactory if leucocyte counts are to be made. For practical purposes it offers no real advantage over the oxalate mixture of Heller and Paul (1934), which is much cheaper, and which, according to Wintrobe and Landsberg (1935), causes no appreciable shrinkage of red cells in the haematocrit tube. This mixture, which consists of potassium oxalate 2 parts and ammonium oxalate 3 parts, was used in the proportion of 2 mg. per ml. of blood in all subsequent experiments where a full blood examination was carried out, the requisite amount of an aqueous solution having been previously measured into the collecting tube and evaporated to dryness.

HAEMOGLOBIN ESTIMATION

The choice of a variety of clinical methods presented itself, ranging from the quick but crude method of Tallqvist (1900) to the quite accurate and simple, but time-consuming, acid haematin method of Sahli (1931). The carboxyhaemoglobin method of Haldane (1901), which is based on the simple dilution technique introduced by Gowers (1879) is, however, both fairly rapid and reasonably accurate, and was selected for these reasons.

The principle of the method of well-known; a standard quantity of blood (0.02 ml.) is drawn into a capillary pipette and transferred to a special graduated tube of about 3 ml. capacity containing a small quantity (not more than about 0.5 ml.) of ammoniated water (0.4% of ammonia, sp. gr. 0.880, in distilled water). The water lakes the blood, forming a solution of haemoglobin, and the mild alkalinity reduces turbidity caused by colloidal protein material. The haemoglobin is then converted to carboxyhaemoglobin by exposing the solution to a brisk stream of coal gas for about 2 minutes, after which the solution is

diluted with more ammoniated water until its colour matches that of a standard solution of carboxyhaemoglobin equivalent to 14.8 g. haemoglobin per 100 ml. ('100% Haldane'). The degree of dilution is then read off on the graduated scale, which is so constructed as to give a direct reading of the haemoglobin content of the sample as a percentage of that of the standard. Multiplying the "per cent. Haldane" reading by 0.148 converts the value to "g. per 100 ml."

As the work progressed, and it was seen that a very large number of haemoglobin estimations would have to be made, the adoption of a still more rapid but accurate method became clearly desirable. Because as many as 150 estimations might have to be made in a single day, it would also be advantageous to make use of a technique which eliminated as far as possible the risk of personal error; the eye fatigue associated with a long day's work, together with changes from morning to afternoon daylight, and finally to artificial light, inevitably affect adversely one's judgment of colour-matches. Accordingly, a photocolorimetric method, making use of an "EEL"* portable absorptiometer, was devised and proved satisfactory. The principle of direct photocolorimetry is well recognised in haemoglobinometry (Sheard and Sanford, 1929; King, 1942; Bell, Chambers and Waddell, 1945; Arnold, 1949; King and Geiser, 1950). It depends on the fact that solutions of oxyhaemoglobin obey Beer's Law, which means (briefly) that they absorb light in proportion to their concentration; therefore, if the proportion of light absorbed by a given solution of oxyhaemoglobin be measured, an estimation of the concentration of the solution can thereby be made. Two methods of doing this are possible: either the amount of incident light entering a solution is varied to give a constant amount of transmitted light, or (the method adopted here) the amount

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* Manufactured by Evans Electroselenium Limited, Harlow, Essex.

of incident light is kept constant, and the extent of light absorption is determined by measuring the amount transmitted. Since a red pigment such as oxyhaemoglobin absorbs green light more readily than any other, increased sensitivity is obtained by restricting the incident light to the green wave-band, and this is accomplished by the use of a suitable green filter. The reading is made from a logarithmically-calibrated scale attached to a galvanometer. The galvanometer is activated by an electric current generated by a photo-electric cell on which the transmitted light is allowed to fall. The logarithmic scale enables direct readings of density to be made, and these are then used to read off the concentration of haemoglobin from a standard curve graph.

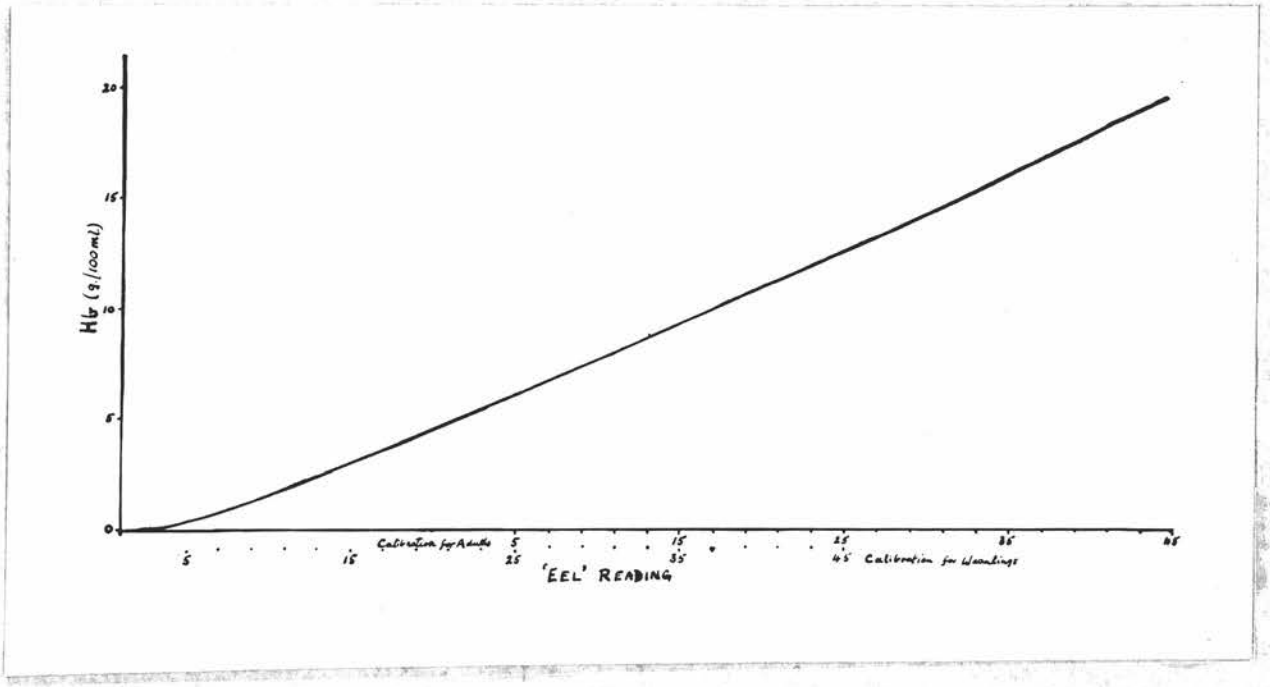
The graph is prepared by holding the incident beam of light fixed at a constant width, and noting the galvanometer readings obtained with various dilutions of a solution of mouse oxyhaemoglobin. These readings are then plotted against the haemoglobin content of the samples (this having been estimated previously by some other method on duplicate samples) and the plotted points are joined by a smooth line. If the width of the incident beam be adjusted initially so that a blank solution of ammoniated water gives a reading of zero on the scale, as has been recommended, then the line will pass through the point of origin of both axes of the graph (i.e. the point $x = 0$, $y = 0$, corresponding to haemoglobin = 0, reading = 0). This means, however, that the more concentrated solutions of oxyhaemoglobin will give a reading high on the galvanometer's logarithmic scale. The calibrations on this scale naturally become progressively closer together as the scale reading increases, and consequently readings obtained in the higher ranges are less accurate than those obtained in the lower ranges. However, if the width of the incident beam be augmented, more light will reach the photo-electric cell, and readings from dense solutions will now fall in the lower part of the scale.

At the same time, readings from weak solutions are now not possible, as they fall below zero on the scale. Therefore, in order that the reading will fall in the lower part of the scale with all solutions, one or two alternatives must be followed: either a single graph is used and the dilution of each sample to be tested is suitably adjusted, a correction for the dilution being made later; or more than one standard graph must be prepared, each corresponding to an incident beam of different width, the appropriate width and graph being selected according to the density of the sample under test. Because it was simpler, and also because it permitted the use of a uniform sampling technique for all specimens of blood, the second of these alternatives was adopted. Two standard graphs were found to be sufficient, one for use with dense solutions (from adult mice) and the other for weaker solutions (from weanlings); and as their slopes were for all practical purposes identical in the middle ranges, it was possible to use a single curve calibrated in two ways (Fig. 37) provided that readings were restricted to these ranges.

As stated above, calibration of the graphs involved the use of solutions of mouse oxyhaemoglobin whose haemoglobin content had previously been determined by some other method. Various accurate methods could have been employed for this estimation, such as the oxygen-combining power method (van Slyke, 1918), the carboxyhaemoglobin method (Haldane, 1901; Palmer, 1918), the alkaline haematin method (Clegg and King, 1942; Gibson and Harrison, 1945), the cyanhaemoglobin method (Stadie, 1920; Evelyn and Malloy, 1938; King and Gilchrist, 1947), the acid haematin method (Sahli, 1931), the pyridine haemochromogen method (Roets, 1940; Rimington, 1942), and the iron content method (Delory, 1943; King, Gilchrist and Delory, 1944). The method selected was again that of Haldane (1901), and for this special purpose, the mean of several estimations was taken. If strictly less accurate than some of the

FIGURE 37

Standard Curve for Oxyhaemoglobin in 'EEL' photoelectric colorimeter, and calibrations of the curve for blood haemoglobin concentration



other methods listed, it is nevertheless quite satisfactory in practised hands, and with strict adherence to a standardised procedure will give results "likely to approximate to the accuracy of more elaborate methods" (Committee on Haemoglobin Surveys, 1945). Macfarlane (1945), who made a thorough statistical study of the errors inherent in the method, states that it "can give results that compare favourably on the point of variability with those of the best modern colorimetric practice". Since I was well accustomed to the method there is little doubt that it gave a more reliable result than would have been obtained with a theoretically more accurate, but unfamiliar technique. Furthermore, its use for this purpose permitted direct comparisons to be made, provided they were otherwise justified, between estimations made photocolorimetrically and others made previously in other experiments.

It may here be remarked that a high degree of accuracy was in fact superfluous in relating galvanometer readings to absolute levels of haemoglobin. Indeed, the purpose of each individual experiment could have been fully served without the establishment of any such relationship at all, the reason being that every experiment was separate and self-contained, the experimentally-fed animals being in every instance compared with litter-mates on control diets. The concern was thus solely with relative as opposed to absolute values. However, it would be possible by statistical manipulation to compare the results of one experiment directly with those of another provided that the units of measurement employed were comparable; the establishment of a constant unit of measurement was therefore desirable, although its relationship to any absolute unit (such as g./100 ml.) did not need to be defined. Even if it were to be objected that only the most strictly accurate absolute values would permit the comparison of my figures with those of another worker, it can be answered that such comparisons would not be valid in any case, as strains of animals, and litters within strains, often vary from each other quite enormously in blood haemoglobin concentration. Between-litter variability is often greater than between-treatment variability, and is certainly much greater than any likely error in the carboxyhaemoglobin method. The preparation of a standard graph relating to very exactly-determined quantities of haemoglobin would therefore have been a quite useless refinement for this type of work.

The use of a photocolorimetric method, as of any method, requires that all variables other than that being measured must be controlled. The

important variables in this case are (1) the intensity of the source of light, (2) the wavelengths passing through the filter, (3) the width of the slit through which the incident light passes, (4) the sensitivity of the photo-electric cell and of the galvanometer recording the electric current generated, (5) the method of preparing the solution to be tested, and (6) the amount of light absorbed by the tubes containing this test solution. The constancy of these variables was ensured as follows:

(1) the mains electricity supply was passed through a voltage-transformer which emitted a steady 2 volts, and the same bulb was used throughout each experiment. (Before the experiment commenced, a supply of spare bulbs was tested for uniformity of intensity in light emission, and held in reserve lest the first bulb should fuse.)

(2) the same filter was used throughout ('EEL' No. 404 green, with peak transmission at about 5300 Å.)

(3) and (4) the constancy of the incident beam and the sensitivity of the cell galvanometer were checked periodically by noting the readings obtained with stable standard solutions of various densities, tested under identical conditions.

(5) the solution to be tested was always prepared in the same way, namely, by diluting 0.02 ml. of whole blood (measured in pipettes standardised at the National Physical Laboratory) to exactly 2.0 ml. with ammoniated water (0.4% ammonia, sp. gr. 0.880, in distilled water), and then transferring it to a test-tube which had been first rinsed with a portion of the solution.

(6) matched test-tubes were employed, each having been previously checked for uniformity in light transmission.

CELL COUNTS AND CELL VOLUME ESTIMATION

The techniques employed for these determinations were all standard procedures, and so do not call for extended description or comment. The same items of apparatus were used throughout each series of observations.

Erythrocyte counts. Thoma pipettes and Neubauer haemocytometers were used, with Hayem's fluid as diluent. The usual dilution employed was 1:200, although for some very anaemic animals it was reduced to 1:100. At least 250 cells were counted in each half of the haemocytometer, and the two counts compared; if they differed by more than 10% (see Holman, 1946) the chamber was reloaded and the counting repeated. In some experiments the diluent used was a 3% aqueous solution of sodium citrate containing 1% commercial formalin (40% formaldehyde) (Dacie, 1950), 0.02 ml. of blood being added to 4 ml. of diluent, thereby obviating the need for Thoma pipettes.

Leucocyte counts. Thoma pipettes and Neubauer haemocytometers were employed, using blood diluted 1 : 20 or 1 : 10 in 1% acetic acid containing a trace of gentian violet. At least 200 cells were counted in each preparation, the maximum permissible difference between the counts in each half of the haemocytometer being set at 10%.

Packed Cell Volume. As the quantities of blood available were very small, Wintrobe's haematocrit tubes could not be used, but graduated capillary tubes proved satisfactory. Estimations were made in duplicate, the tubes being tightly sealed at both ends by means of a rubber-lined metal casing, and centrifuged for 1 hour at 3000 r.p.m. (R.C.F. = 1500 g.) The maximum permissible difference between duplicates was set at 1% of the height of the original column of blood.

ERYTHROCYTIC INDICES.

Blood haemoglobin concentration (Hb) was measured, in the first instance, as a percentage of the Haldane standard, which is equivalent to 14.8 g. haemoglobin per 100 ml. of blood. These percentage measurements were then transformed into absolute units by multiplying the percentage by 0.148. Thus 50% Haldane would be equivalent to 50 x 0.148 = 7.40 g. haemoglobin per 100 ml. of blood. Red blood cells (R.B.C.) were measured as millions per cu. mm. of blood, and packed cell volume (P.C.V.) as the percentage volume of cells per volume of blood.

These three observed values, in the units stated above, were used to calculate the three erythrocytic indices M.C.H., M.C.H.C., and M.C.V.

The mean cell haemoglobin (M.C.H.) measures the mean weight of haemoglobin per red cell. The unit of measurement is μμg., and the formula for its calculation is: $\frac{\text{Hb x 10}}{\text{R.B.C.}}$.

The mean cell haemoglobin concentration (M.C.H.C.) describes the average degree of saturation of the red cells with haemoglobin. It measures the w/v percentage of haemoglobin per unit volume of red cells, and is derived as follows: $\frac{\text{Hb x 100}}{\text{P.C.V.}}$.

The mean cell volume (M.C.V.) is a measure of the ratio of red cell volume to red cell number. The unit of measurement is the cμ, and the formula is:

$\frac{\text{P.C.V. x 10}}{\text{R.B.C.}}$.

2. HISTOLOGICAL METHODS

Fixation. Except for special purposes, tissues were fixed in either 4% (w/v) neutral formol-saline (10 parts commercial formalin, i.e. 40% formaldehyde, in 90 parts normal saline, an approximately neutral reaction being maintained

by the addition to the stock solution of a little calcium or magnesium carbonate), or in formol-corrosive (10 parts commercial formalin in 90 parts saturated aqueous solution of mercuric chloride) (Lendrum, 1943). Where tissues might have to be stored for several weeks before being processed, neutral formol-saline was preferred. Bone marrow tissue was fixed in the modified Zenker-formol solution of Cappell, Hutchison and Smith (1947).

Paraffin Sections. The tissues were dehydrated in progressively stronger solutions of ethyl alcohol (70%, 95% with 6% phenol, 100% and 100%), cleared in chloroform and then in benzol, and finally impregnated with, and embedded in, paraffin wax (M.P. 56° C.) containing 2% beeswax. The chief deviation from standard histological procedure was the short time allowed to each of these operations, particularly the impregnation with molten wax. It was found that mouse tissue, especially the tiny organs of 21 day-old mice, were extremely liable to become distorted and damaged during processing; with such material, best results were obtained by completing the entire procedure from dehydration to embedding within 7 hours, with no more than a total of 45 minutes in the two impregnation baths. Sections were cut in the usual way and, after staining, were mounted in DePeX.*

In processing bone marrow, the method followed was that advocated for sternal puncture material by Cappell, Hutchison and Smith (1947).

Frozen Sections. Frozen sections were cut in the usual way on a freezing microtome cooled by subliming carbon dioxide. After staining they were mounted in glycerine jelly.

Staining. Where tissue had been fixed in formol-corrosive, the mercury was removed by treating sections before staining with 0.5% iodine in 95% alcohol, followed by 2% aqueous solution of sodium thiosulphate. For routine purposes,

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* Messrs. George T. Gurr, Ltd., London.

all paraffin sections were stained by Mayer's (1903) haemalum and eosin. Fat in frozen sections was stained by Scharlach R, with haemalum as the counterstain. Fresh films of blood or bone marrow were stained by Leishman's (1901) method. Reticulocytes were demonstrated in coverslip preparations by supravital staining with 0.3% brilliant cresyl blue, the film being counterstained with Leishman's stain.

3. CHEMICAL METHODS

Serum Calcium. The method employed was based on that of Kramer and Tisdall (1921), as modified by Clark and Collip (1925), and was as follows:

2 ml. serum and 2 ml. half-saturated sodium oxalate solution were mixed together in a centrifuge tube and allowed to stand overnight. After centrifuging the tube for 10 minutes at 2000 r.p.m. (R.C.F. = approx. 675 g) the supernatant fluid was carefully decanted, and the tube, while still inverted, placed in a rack to drain for 5 minutes, the mouth of the tube resting on a pad of filter paper. The inside and mouth of the tube were then dried with filter paper, and the precipitated calcium oxalate and the walls of the tube washed with 3 ml. dilute ammonia solution (2% of 0.880 ammonia) directed in a fine stream. The centrifuging, draining and drying procedures were then repeated, and finally the precipitate was broken up by blowing on to it 2 ml. of approximately $\frac{N}{10}$ sulphuric acid. When the precipitate had completely dissolved, the tube was warmed in a beaker of water at 70° C. After 1 minute at this temperature, titration was commenced from a 2 ml. micro-burette with approximately 0.01 $\frac{N}{10}$ potassium permanganate, while maintaining the temperature of the tube at 70° C. The permanganate solution was freshly diluted before use from a stock 0.1 $\frac{N}{10}$ solution, and the end-point was taken as the point where a pink tinge persisted for 30 seconds. A blank, using distilled water in place of serum, was run in

duplicate to determine the titration value of the reagents employed, this value being subtracted from the observed titration reading. The reading was further adjusted to compensate for the amount by which the permanganate solution differed (as determined by titrating against a standard sodium oxalate solution) from exactly 0.01 $\bar{N}$.

By this method, the number of milligrams of Ca in 100 ml. serum is given by 10 times the number of millilitres of 0.01 $\bar{N}$ potassium permanganate required.

Serum Inorganic Phosphate. The method described by King (1946) was employed to convert inorganic phosphate into a blue solution with a colour density directly proportional to the amount of phosphate present. Details of the procedure are given by that author, and will not be transcribed in full here. Briefly, 0.2 ml. serum (free from haemolysed cells) was deproteinised with trichloroacetic acid, and a filtrate prepared. Acid ammonium molybdate was added to a portion of the filtrate, and reduction of the resultant yellow phospho-molybdate was then effected with a reducing agent containing 1:2:4 aminonaphtholsulphonic acid, sodium metabisulphite and sodium sulphite. A blue colour developed, whose density was read after the solution had stood for exactly ten minutes.

The instrument used for measuring the density of colour in the solution was a 'Spekker' photoelectric absorptiometer,* fitted with an orange-red filter. A standard curve was prepared by making a series of dilutions from a standard solution of potassium di-hydrogen phosphate, and, after the phosphate had been converted into the soluble blue compound, plotting the phosphate content of the standard solutions against the light absorption readings given by them on the 'Spekker'. Each reading was, of course, first corrected for the reading given by a reagent blank. Using this standard curve, the concentration of

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* Messrs. Hilger and Watts Ltd., London.

phosphate in an unknown solution could be read off against its light absorption reading.

Estimation of serum inorganic phosphate was invariably made on the same day as the blood had been collected, to guard against the possibility of enzymatic hydrolysis of ester phosphate. For the same reason the serum was stored in a refrigerator between the times of separating it from the blood clot and beginning the estimation. Whenever the quantity of serum available was sufficient, estimations were made in duplicate. The accuracy of the instrument, and the efficacy of the reagents, were checked on each occasion of their use by running a reagent blank and a standard phosphate solution along with each batch of serums.

Serum lipoids. The methods employed were based on those advocated by Peters and van Slyke (1932).* Proteins were precipitated and lipoids extracted from the serum by means of a boiling alcohol-ether mixture (Bloor, 1914), which was then filtered through fat-free filter paper. Aliquots of this filtrate were used to determine total fatty acids by the saponification and titration method of Stoddard and Drury (1929). The ether was first driven off by gentle heat, and the fatty acids saponified by boiling with sodium hydroxide. The mixture was then evaporated to dryness after just restoring its reaction to alkalinity. The soaps were dissolved in water, and their fatty acids released by treatment with dilute hydrochloric acid. The fatty acid suspension thus formed was filtered through a dense paper pulp emulsion contained in a small Gooch crucible, and washed repeatedly with sodium chloride solution (neutralised to methyl red) until the filtrate showed no trace of acid. In this way, the fatty acids were retained in the crucible, and were then dissolved in hot alcohol. Suction was applied, and the solution was collected

* On the recommendation of Dr. J. Duckworth, Head of the Section of Applied Biochemistry, Rowett Research Institute.

in a graduated test tube. After the solution had been boiled for one minute its volume was noted, and it was then titrated against a standard solution of sodium hydroxide, with thymol blue as the indicator. An equal volume of boiled alcohol was similarly titrated as a blank.

As only small quantities of serum were available, quantitative alterations were made to the recommended methods. These involved the use of 3 ml. instead of 5 ml. of serum, extracted with 50 ml. instead of 100 ml. of alcohol-ether mixture. An aliquot of 30 ml. instead of 75 ml. of the filtered extract was taken, and the free fatty acids were titrated with 0.01 $\overline{N}$ instead of 0.02 $\overline{N}$ sodium hydroxide. Consequently, the formula for fatty acid concentration quoted by Peters and van Slyke (1932) had also to be modified; calculation showed that milligrams of fatty acid per 100 ml. serum were given by, not 147A-148B, but 152A-154B, where A represents the number of millilitres of alkali used in the titration, and B the number of millilitres of alkali used to titrate the blank.

At a later stage of the work, when the adoption of a more rapid micro-technique became desirable, the literature was searched for suitable methods. Among the possibilities considered were various techniques depending upon the conversion of the fatty acids to carbon dioxide, and its subsequent measurement in the van Slyke apparatus (Page, Kirk, Lewis, Thompson and van Slyke, 1935); the method of Smith and Kik (1933); gravimetric methods such as those of Elkes, Frazer and Stewart (1939) and Nadeau (1951); the colorimetric method of Bauer and Hirsch (1949); the lipo-micrograph technique of Frazer and Stewart (1939); and the "haemolipokrit" method of Rückert (1931), as used also by Collins (1933) and - in a slightly modified form - by Hermann, Ames and Tapke (1933-4). These methods all had disadvantages, and the method finally selected was a turbidometric one. Geyer, Mann and Stare (1948) have described a turbidometric method for whole blood, but my choice fell on the

method advocated by Kunkel and Ahrens (1948) for the total lipids of serum.

This method is both simple and rapid and, as the turbidity of the serum is directly proportional to the concentration of total serum lipid, and only relative values were required, the necessity of preparing a standard curve was avoided. Before the degree of turbidity was measured (in an 'EEL' photometer, at about 650 mμ), the serum was diluted in 12% aqueous sodium chloride solution. A dilution of 1 in 15 was found suitable, as with this strength of serum the readings fell within the more sensitive part of the galvanometer scale. 3 ml. of diluted serum was ample for the small 'EEL' tubes, and thus readings could be made from as little as 0.2 ml. of serum (pooled from each litter). As there was no interference from colloidal proteins - these having been salted out by the hypertonic salt solution - the turbidity could be assumed to be due entirely to the lipid content of the serum.

E. INTERPRETATION OF CHANGES IN BLOOD HAEMOGLOBIN CONCENTRATION

The interpretation of much of the experimental work described in this thesis depends on the assumption that improved absorption or utilisation of iron will be reflected in a proportionately higher blood haemoglobin level. This principle has been the subject of much debate in the past, and although the controversy has now been resolved, in view of its great importance to the present work it deserves consideration in some detail.

> Amongst those who have questioned the validity of the assumption are Fowler and Barer (1935, 1937a, b, 1940), Brock (1937a), Brock and Hunter (1937) and Dubach, Callender and Moore (1948). The views of this group were clearly expressed by Moore, Arrowsmith, Welch and Minnich (1939), who pointed out that the use of the haemoglobin level as an index of iron absorption is an indirect measure, and could be challenged "on the ground that much more iron is actually absorbed and retained by the body following therapeusis than is utilised for immediate haemoglobin synthesis." On the other hand, it is admitted - even by some of the workers quoted - that the utilisation of absorbed iron in

anaemic iron-deficient subjects is complete and Finch, Gibson, Peacock and Fluharty (1949) have stated that it is also more rapid. With anaemic rats, Elvehjem (1932a) found that provided haemoglobin formation was not hindered in other ways, such as by copper deficiency, the increase in haemoglobin level following the administration of iron was proportional to the iron intake, and Josephs (1932) concluded from his experiments that the proportion of absorbed iron appearing in the blood of anaemic animals was practically 100%. Sherman, Elvehjem and Hart (1934) went on to show also that when various foodstuffs containing iron were fed, haemoglobin regeneration in anaemic rats was proportional in each case to the amount of iron in the diet. In agreement with this finding, Whipple and Robscheit-Robbins (1936) reported that iron injected into anaemic dogs could be accounted for quantitatively by the new haemoglobin formed; Heath, Strauss and Castle (1932) came to the same conclusion. More recently, Dubach, Moore and Minnich (1946), using radio-active iron, found that utilisation of injected iron in iron deficiency was 'prompt and complete'. Dubach and her colleagues also put forward evidence that recently acquired iron is held in a more readily mobilisable form than is the iron in body stores of longer standing, and that it is used selectively for haemoglobin production; from this it follows that blood haemoglobin is likely to be a better index of recent iron absorption than would otherwise be the case. Others whose work supports these findings include Reimann, Fritsch and Schick (1937), Hahn, Bale, Lawrence and Whipple,(1938), Hahn, Ross, Bale and Whipple (1940), and Copp and Greenberg (1945).

It is clear, however, that the haemoglobin level itself does not control iron absorption; the most important controlling factor is probably the state of the body iron stores (Chapin and Ross, 1942; Hahn, Bale, Ross, Balfour and Whipple, 1943; Cartwright, Wintrobe and Humphreys, 1944; Chodos, Ross, Adams and O'Brien, 1952), and there is also a sex difference (Mitchell, 1932a, b; Steenbock, Semb and van Donk, 1936; Smith and Otis, 1937a, b; Hubbell and Rose, 1937; Mitchell and Hamilton, 1937).

The position thus seems to be that the haemoglobin level is a good index of iron utilisation in anaemic animals, although it should not be used in comparisons between animals of different sex and cannot be used as such in animals whose haemoglobin level is already high. Indeed, this is not to be wondered at, and the situation conforms with the 'room for improvement' principle which is elaborated elsewhere in this thesis (p. 86). Consequently, the use of the haemoglobin level in this way is still accepted as a valid procedure and has been used not only in the past (e.g. Mitchell and Vaughn, 1927), but also in more recent years (Nakamura and Mitchell, 1943; Street, 1943; Freeman and Burrill, 1945) for testing the availability of iron given in various

forms and under various dietary conditions. (For special purposes, however, or when fuller information is required, other techniques may be preferable, such as estimation of total carcase iron (Kletzien, 1940; Spray, 1950) or of serum iron concentration (Moore et al., 1939; Nordensen, Rydin and Sandell, 1949), or the use of radio-active iron (Hahn, Bale, Lawrence and Whipple, 1939).)

It may safely be concluded that so long as the initial haemoglobin level and the iron stores are low, and comparisons are restricted to members of the same sex, it is justifiable to assume - as was done in my experiments - that improved utilisation of iron will be reflected in a proportionately higher blood haemoglobin level.

F. EXPERIMENTAL EFFECTS OF ETHER ANAESTHESIA AND STUNNING ON HAEMOGLOBIN CONCENTRATION

EXPERIMENT 13

1. Introduction

It has been indicated (p. 248) that stunning young mice by means of a sharp blow on the back of the head might have certain advantages over ether anaesthesia as a method of preparing them for the collection of blood samples, but also that it was doubtful whether the two methods would give comparable results.

A review of the literature shows that there has been considerable controversy on the effects of ether anaesthesia on the concentration of the cellular elements of the blood.

Barbour and Bourne (1923-24) noted haemoconcentration after ether anaesthesia in dogs, and they quote earlier work leading to the same conclusions. Bollman, Swirbley and Mann (1938) found that ether anaesthesia decreased plasma volume by 6% in the dog, with a resulting increase in the haematocrit reading. McAllister (1937, 1938), injected a blue dye into the blood stream of dogs, and found a decrease in plasma volume of 11%, and also a rise in plasma proteins and in cell volume. Jarcho (1943), also working with dogs, reported that the haemodilution associated with nembutal anaesthesia was more than overcome

by the subsequent administration of ether, the volume of red cells and the concentration of plasma protein rising above control levels. In man, Searles (1939) noted increases in the haematocrit reading, red cell count and haemoglobin concentration following ether anaesthesia. Smith, Oster, Snyder and Proutt (1948) observed similar effects in the cat, the haematocrit rising by about 30% and plasma protein concentration by about 6%. The latter workers are among those who attribute much of the haematocrit rise following the administration of ether to the action of the spleen, since in more than one species this rise has been shown to be markedly less in splenectomised than in intact animals. This could be explained by the fact that the spleen is an important depot in which reserves of red cells are held (Barcroft and Barcroft, 1923-24), but the action of this organ alone does not seem capable of explaining a drop in plasma volume. Possibly the adrenal gland is implicated, since McAllister and Thorn (1937) have claimed that the blood volume may be preserved by the repeated intravenous injection of an adrenal cortical extract.

But, on the other hand, Conley (1941) could find no change in plasma volume, protein concentration or haematocrit reading in ether-anaesthetised cats. The findings of Crafts (1944) were somewhat similar; he reported that ether anaesthesia had no effect on the concentration of either red or white blood cells in the cat, the rabbit or the rat, although he did observe haemoconcentration in the dog. This report does not conform with the statement of Smith et al. (1948) that "there is general agreement that inhalation anaesthesia increases, and barbiturates act to decrease, the white cell count". These workers found the rise in white cells substantially similar to the rise in red cells; and Cushnie (private communication) is another who has noted a quite appreciable difference in the leucocyte counts of rats before and after ether anaesthesia.

In man, Stewart and Rourke (1938) found an average reduction of 14.9% in plasma volume after the administration of ether, but did not observe corresponding increases in the concentration of many blood solids, including red cells and plasma proteins. They therefore pointed out that it is fallacious to assume that haemoglobin concentration and packed cell volume necessarily change with alterations in plasma volume. Kohn (1950) is another who has reported, this time with the rat, that ether anaesthesia does not affect the level of haemoglobin or blood protein.

It seemed possible that species differences might have accounted in part for the conflicting nature of these reports. No previous work appears to have been recorded on the effects of ether anaesthesia on the blood of mice, nor have I been able to trace any literature dealing with the effects of stunning on haemoglobin concentration. This experiment was therefore undertaken with the object of comparing the effects of ether anaesthesia and

stunning on the haemoglobin concentration of the blood of weanling mice.

2. Methods

It was obviously impossible to impose both variables (stunning and ether anaesthesia) successively on the same experimental animals, and consequently littermate weanlings were employed. The animals used had been bred from mothers on various diets, and were drawn from nine litters. Litters were sorted into sexes and then littermates of similar sex were assigned equally, but at random, to two groups. Although the numbers in each group varied from litter to litter, nevertheless the two groups were strictly comparable within each litter (except where the number of one sex in a litter had been odd, in which case one of the groups contained an extra animal).

The animals in one group were stunned, and those in the other anaesthetised with ether, immediately before the withdrawal of a blood sample whose haemoglobin concentration was subsequently measured by photocolorimetry.

3. Results

The results are shown in Text Table 80. The litter means are unweighted, except with those litters where the numbers of animals in the two groups were unequal. In these cases, the additional accuracy provided by the extra animals was utilised, but any possible influence of sex was eliminated by first calculating a sex mean for the sex containing the supernumerary animal (where this was done, the values are shown in parentheses in the Table). For example, in litter 5 there is an extra male in the stunned group and an extra female in the etherised group; instead of taking the simple arithmetic mean of the four heterogeneous animals in each group, the mean of the stunned group was found from $\frac{\frac{1}{2}(7.10 + 6.73) + 7.18 + 5.92}{3}$, and that of the etherised group

TEXT TABLE 80.

Exp. 13. Hb (g./100 ml.)

Litter No.	STUNNED GROUP						ETHERISED GROUP					
	M.	M.	F.	F.	F.	Litter-Mean	M.	M.	F.	F.	F.	Litter-Mean
1	4.51	3.85	4.51	5.11		4.50	3.70	3.70	3.85	4.37		3.91
2			7.10	8.29	7.25	7.55			7.10	7.40	7.25	7.25
3	4.81	6.22	4.96	5.48		5.37	4.96	4.66	4.96	5.33		4.97
4	2.22		2.52	3.11	2.81	2.66	2.81		2.52	4.00	3.11	3.11
5	(7.10)	(6.73)	7.18	5.92		6.67	5.92		(5.33)	(4.81)	(5.33)	5.42
6	(3.11)	(1.33)	2.96	1.92		2.37	1.85		2.07	2.22		2.04
7	5.92	6.36	6.22	5.33		5.96	4.66	5.33	6.07	5.11		5.30
8	(3.40)	(4.37)	4.07	4.81		4.26	3.70		3.77	4.00		3.82
9	(3.70)	(4.00)	3.70	3.55		3.70	2.81		(3.70)	(3.55)	(2.96)	3.21
				Group Mean		4.78				Group Mean		4.34

Standard error of the difference between the group means = ± 0.147

from $\frac{5.92 + \frac{2}{3}(5.33 + 4.81 + 5.33)}{3}$, thus making each group equivalent to one male and two females. By this means, homogeneity was preserved between the two groups so that they remained strictly comparable.

When the difference between the group means was analysed statistically it was found to be significant ($P < 0.02$), the mean of the stunned group being the higher.

4. Discussion

The work quoted in the introduction to this section had suggested that ether anaesthesia might have increased the blood haemoglobin concentration. The result of this experiment does not necessarily conflict with this possibility, but it shows that stunning certainly acted in this way and that, even if ether had also done so, the effect of stunning had been greater than that of ether. Although the difference between the two groups was comparatively small (averaging only 0.44 g./100 ml., or 3% Haldane) it was nevertheless constant enough to show that stunning and ether anaesthesia should not be used interchangeably in preparing young mice for blood sampling.

This experiment does not, of course, indicate the reason for the increase in haemoglobin concentration following stunning, but it may be speculated that it resulted from haemoconcentration following loss of vagal function. In this connection, it will be recalled that the rise observed by some other workers following ether administration has also been attributed to haemoconcentration, and it might here be suggested that this could result from sympathogotonia associated with the excitement and fear frequently generated by the induction of inhalation anaesthesia.

By the method I employ, the induction of anaesthesia is normally rapid and proceeds with the minimum of distress to the animal; but with one litter in this experiment - litter 4 - it was remarked at the time that, owing to the

concentration of ether vapour having become too low, induction was slow and that consequently there had been an unusual degree of excitement and struggling. It is therefore interesting to note that this particular litter provided the only instance where the mean of the etherised group exceeded that of the stunned group, and it is tempting to suggest that the excitement factor was the cause of this relatively high value. On the other hand, the explanation may be simply that the longer period required in this case for anaesthesia to reach the requisite depth allowed more time for the full development of haemoconcentration brought about in some other way.

APPENDIX II

BIBLIOGRAPHICAL APPENDIX

BIBLIOGRAPHY

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APPENDIX III

TABULAR APPENDIX

APPENDIX TABLE I

Exp. 1. Litters

Dam's Litter No.	Row	Column	Sex	Hb	R.B.C.	P.C.V.	W.B.C.
P	1	1	M	20	...	...	
			M	26	...	...	
			F	21	...	...	
			F	19	...	...	
Q	2	3	M	32	...	...	
			F	28	...	...	
			F	26	...	...	
			F	30	...	...	
			F	20	...	...	
			F	27	...	...	
S	3	2	M	33	...	...	
			M	46	...	...	
			M	38	...	...	
			M	40	...	...	
			F	40	...	...	
C	4	4	M	<20	...	...	
F	3	4	M	26	...	...	
			F	30	...	...	
H	1	4	M	22	...	...	
			F	22	...	...	
			M	24	...	...	
			M	22	...	...	
			F	22	...	...	
S	2	4	M	26	...	...	
			M	28	...	...	
M	2	2	M	20	...	...	
			F	<20	...	...	
			F	<20	...	...	
K	3	2	F	46	...	...	
			F	50	...	...	
			F	47	...	...	
			F	52	...	...	
			F	50	...	...	
K	1	4	M	20	...	...	
			M	26	...	...	
R	4	2	M	46	...	...	
			M	43	...	...	
			M	52	...	...	
			M	52	...	...	
			F	40	...	...	
E	4	4	M	27	...	...	
			F	21	...	...	
L	1	4	M	36	...	...	
			M	28	...	...	
			M	32	...	...	
R	1	3	M	22	...	...	
			F	26	...	...	
			M	24	...	...	
			F	22	...	...	
			M	22	...	...	
			F	26	...	...	
N	3	3	M	22	...	...	
			M	<20	...	...	
			M	20	...	...	
N	4	1	M	38	...	...	
			F	39	...	...	
			F	38	...	...	
			M	36	...	...	
			F	34	...	...	
			M	48	...	...	
M	1	3	F	<20	...	...	
			F	<20	...	...	
H	2	3	M	48	...	...	
			M	41	...	...	
			F	49	...	...	

(continued)

(continued)

APPENDIX TABLE I

Exp. 1. Litters

Dam's Litter No.	Row	Column	Sex	Hb	R.B.C.	P.C.V.	W.B.C.
G	3	2	M	46	...	...	
			M	50	...	...	
			F	46	...	...	
			F	40	...	...	
			F	54	...	...	
C	1	2	M	23	...	...	
			M	25	...	...	
			M	24	...	...	
L	3	2	F	36	...	...	
			F	36	...	...	
			F	31	...	...	
			F	47	...	...	
K	2	1	F	60	...	...	
			F	73	...	...	
			M	70	...	...	
			M	62	...	...	
D	2	4	M	26	3.5	...	
			M	28	...	...	
			F	24	...	...	
			F	24	...	...	
G	4	4	F	<20	2.4	...	
			M	<20	...	...	
			F	<20	1.5	...	
H	3	1	F	52	...	...	
			M	47	...	...	
			F	46	7.1	...	
			M	51	6.3	...	
N	1	4	M	28	4.8	...	
			F	23	2.7	...	
			F	26	...	...	
			M	26	...	...	
H	4	2	M	48	6.7	...	
			M	48	5.3	...	
			F	44	...	...	
			M	62	...	...	
			M	52	...	...	
			F	52	8.2	...	
			F	49	...	...	
			F	51	6.7	...	
Q	4	1	F	40	...	...	
			M	34	...	...	
			M	45	...	...	
			F	40	...	...	
A	3	4	M	32	5.3	...	800
			M	34	...	...	
			F	40	...	...	
			M	32	5.1	...	1,200
			M	39	...	...	
			F	45	...	...	
B	2	4	F	36	4.4	...	300
			F	29	4.1	...	1,600
			F	30	...	...	
			M	30	...	...	

(continued)

(continued)

APPENDIX TABLE I

Exp. 1. Litters

Dam's Litter No.	Row	Column	Sex	Hb	R.B.C.	P.C.V.	W.B.C.
A	1	2	F	65	8.8	...	1,800
			M	60	8.9	...	1,000
			F	58	...	...	
			F	54	...	...	
A	4	3	M	54	7.8	...	
			F	42	6.7	...	
			F	55	...	23	
			F	56	...	19	
L	4	1	F	66	8.2	19	2,600
			M	64	8.0	...	1,600
			F	69	...	...	
			F	72	...	...	
F	4	2	M	38	6.6	16	700
			F	40	4.8	17	1,200
			M	36	...	...	
			M	36	4.7	...	
E	4	3	M	44	6.1	23	700
			M	45	7.5	22	2,300
			F	47	7.3	24	1,300
			M	43	...	...	
F	2	1	F	46	6.0	21	800
			M	50	5.8	23	1,200
			F	45	5.9	21	500
			M	46	...	...	
F	1	3	M	44	7.0	29	1,700
			F	38	5.6	20	900
			M	43	5.8	...	700
			M	42	...	...	
B	3	1	M	18	2.6	18	400
			F	18	2.8	12	2,500
			F	25	...	...	
			M	18	...	...	
S	2	4	F	32	5.2	12	600
			F	33	5.5	18	400
			F	36	...	...	
			M	28	4.9	16	100
C	3	3	F	18	1.9	6	700
			F	16	2.0	6	700
			F	20	...	...	
			F	16	2.1	...	1,000
N	2	2	F	<20	2.2	8	400
			M	<20	1.9	...	400
			F	<20	2.6	6	100
			F	<20	...	...	
M	1	3	F	<20	2.8	12	700
			F	<20	2.2	10	600
			M	22	3.5	14	200
			M	20	2.8	12	100
K	1	4	F	23	3.6	13	700
			F	22	3.2	10	200
			M	26	4.5	12	700
			M	22	3.2	13	800
N	4	1	M	29	4.0	12	2,100
			M	20	2.3	9	600
			F	22	3.1	8	900
			F	24	3.2	12	2,600
			M	20	2.7	10	2,800
			M	24	3.6	15	1,200

APPENDIX TABLE II

Exp. 1. Dems

Litter	Row	Column	Hb	R.B.C.	P.C.V.	W.B.C.
C	2	1	87	10.4	...	...
B	4	3	50	7.0	...	...
L	2	3	94	12.5	...	...
D	2	4	90	9.6	...	...
A	2	1	108	9.1	...	...
B	1	2	94	6.8	...	...
D	4	2	102	10.0	...	400
H	4	2	98	9.2	...	3,800
D	3	3	110	11.3	...	4,200
M	4	4	95	11.6	...	5,300
H	3	1	102	11.0	...	2,000
N	3	3	80	12.1	...	950
N	1	4	70	8.7	...	1,300
B	2	4	93	12.2	...	...
A	1	2	94	10.1	39	1,900
F	2	2	97	12.1	47.5	550
E	2	2	94	8.5	38	5,300
L	4	1	115	11.7	43	10,000
F	4	2	91	11.5	31	2,100
R	4	2	110	10.2	38	5,000
H	2	3	94	10.4	35	600
Q	2	3	98	10.9	36	2,200
K	2	1	104	9.3	54	1,400
C	3	3	78	10.0	43	1,800
S	2	4	84	10.8	47	1,600
N	2	2	59	8.6	23	500
B	3	1	118	15.5	55	1,900
F	1	3	98	10.6	54	7,300
F	2	1	98	9.1	44	3,400
Q	1	2	100	15.9	...	3,600
C	1	2	93	8.8	...	1,400
P	1	1	104	11.8	48	2,200
D	1	1	96	8.6	45	1,700
E	1	1	102	10.5	47	1,000
P	4	3	108	11.3	46	2,600
A	4	3	118	8.7	55	4,400

(continued)

(continued)

APPENDIX TABLE II

Exp. 1. Dams

Litter	Row	Column	Hb	R.B.C.	P.C.V.	W.B.C.
K	4	3	90	12.8	37	400
R	3	1	106	13.8	49	1,500
P	3	4	90	11.0	38	5,000
F	3	4	84	10.6	43	1,500
A	3	4	99	11.1	47	2,000
Q	3	4	94	10.0	44	1,000
G	3	2	100	11.4	46.5	4,200
S	3	2	98	10.5	47	2,700
K	3	2	103	8.2	46	1,000
C	4	4	37	6.2	24	200
G	4	4	79	10.1	42	1,300
E	4	4	90	10.2	45	2,000
H	1	4	84	11.4	50	3,300
L	1	4	93	13.0	48	4,000
Q	4	1	109	13.2	53	900
R	1	3	90	11.2	48	1,600
S	4	1	100	9.0	47	3,400
S	1	3	104	11.5	46	2,800
M	1	3	87	11.4	42	...
K	1	4	74	9.2	33	400
N	4	1	88	13.3	12	2,600
E	3	3	115	13.0	40	1,800

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The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

1. Effects on Reproduction and on the Heart and Thymus Weights of the Weanlings

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Within recent years several experiments on laboratory animals at this Institute have indicated that additions of calcium carbonate to the diet may have deleterious effects. Thus the effects of pyridoxin deficiency induced in suckling rats by excess of aneurin were accentuated when calcium carbonate was also added to the diet (Richards, 1945); when the amount of added $CaCO_3$ was increased, there was also a retardation in the rate of growth of young rats after weaning (Richards, 1949*a*). Simpson (1947) found that additions of $CaCO_3$ to the diet of adult rats increased iodine excretion and diminished the iodine content and concentration in the thyroid. More recently Howie & Porter (1950) found that a slight modification of the B diet of Sherman gave better reproductive results in mice than another modification in which one of the changes was a 1% addition of $CaCO_3$.

These observations led to the present experiments, of which the initial object was to test the effects on reproduction in mice of including different levels of $CaCO_3$ in the original slightly modified Sherman B diet and at the same time to assess the relative importance of the various changes made in this diet by Howie & Porter (1950). The results of the first experiment indicated that any differences resulting from the other modifications were of minor importance compared with those due to the additions of $CaCO_3$. A second experiment was therefore designed to investigate means by which the deleterious effects of these additions could be counteracted. As the work developed, observations were made indicating that it would be of interest to record the weights of various organs of the young mice and their mothers. These aspects are best considered separately, and the present paper is accordingly presented in two parts: the first deals with reproductive effects and the second with effects on organs.

1. EFFECTS ON REPRODUCTION

Two experiments are reported. Materials and methods were similar for both and are described together, but designs and results are given separately for each experiment.

MATERIALS AND METHODS

Both experiments were done with white 'Swiss' mice of the Institute's stock colony. To supply the breeding females for each experiment sufficient litters containing four females were bred at the same time. They were bred on stock diet (diet 61), which

consisted of Rowett Institute stock cubes (diet 1 of Howie & Porter, 1950) with a daily supplement of fresh whole milk given *ad lib.* to the mated pairs of adult mice. Four female mice were allotted to each group in Exp. 1 and eight in Exp. 2 with randomization of litter-mates. Each breeding female was reared on diet 61 to the age of 7 weeks and was then given the appropriate experimental diet a week before being mated with a male of the same age. The males were reared in the same way as the females; mating of litter-mate brothers and sisters was avoided. The method of continuous monogamous mating was used, the effect of the diets on reproduction being judged by the number and weight of the young reared in a given period, as described by Bruce & Emmens (1948). In Exp. 1 the males were removed from the breeding cages after 80 days, and the females were maintained on the diets long enough to rear any litters that had been conceived. In Exp. 2 sufficient time was allowed for the rearing of only two litters. The observations were ended by killing the litters on weaning at 21 days of age. Post-mortem examinations were made as detailed in Part 2 of this paper.

EXPERIMENT 1

The object of Exp. 1 was to test on reproduction in mice the effect of adding three levels of $CaCO_3$ to the slightly modified Sherman B diet used by Howie & Porter (1950)—their diet 2— and to three further modifications of it. Diet 2 consisted of: ground whole wheat 66, dried whole milk 33 and NaCl 1 %. Diet 5—the modified diet that gave poorer reproductive results than diet 2 in their experiments—contained: ground whole wheat 60, casein (lactic, unextracted, Glaxo Laboratories Ltd.) 5, dried whole milk 33, $CaCO_3$ 1 and NaCl 1 %. The most likely causes of the poorer results with diet 5 seemed to be either the addition of $CaCO_3$ or a deficiency of the vitamin B complex. Two factors might have contributed to such a deficiency, namely the reduction in the proportion of wheat and the increased requirement for vitamin B resulting from the addition of protein as casein. These possibilities were therefore considered in planning the experiment.

Design

A factorial design of sixteen squares was drawn up, as shown in Table 1, each square representing an experimental diet. Each of the four rows was built upon a different basal diet. In the first row the basal diet was diet 2. In the second row the basal diet (diet 70) was formed from diet 2 by substituting 6 g maize starch for 6 g wheat, thus reducing the vitamin B content without increasing the protein. In the third row the basal diet (diet 74) was derived from diet 2 by substituting 6 g casein for 6 g wheat, thus reducing the vitamin B content but this time increasing the protein. Rows 1, 2 and 3 were completed by the addition of $CaCO_3$ at the rate of 0·5, 1·0 and 2·0 g/100 g of the respective basal diets. In the fourth row 5 g casein were added to 100 g of diet 2 to form diet 78, and the same additions of $CaCO_3$ were made to 105 g of this basal diet. The number in each square of the plan in Table 1 indicates the number of the diet and is used also to designate the group of experimental animals fed on the diet.

Table 1. *Exp.* 1. *Plan of dietary groups to show factorial design*

(The numbers within the table refer to diets. They are also used in the text to designate the corresponding groups of experimental animals)

Row no.	Composition of basal diet for each row, i.e. the diets for column 1		Amount of added $CaCO_3$ (g/100 g basal diet in rows 1–3; g/105 g in row 4)			
	Constituent	Parts	0 (column 1)	0·5 (column 2)	1·0 (column 3)	2·0 (column 4)
1	Ground whole wheat	66	2	67	68	69
	Dried whole milk	33				
	NaCl	1				
	Total	100				
2	Ground whole wheat	60	70	71	72	73
	Maize starch	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
3	Ground whole wheat	60	74	75	76	77
	Casein	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
4	Ground whole wheat	66	78	79	80	81
	Casein	5				
	Dried whole milk	33				
	NaCl	1				
	Total	105				

Results

First litters. A summary of the reproductive results for the first litters born to each breeding female is given in Table 2. In the upper part of the table figures are given for the separate groups according to the plan shown in Table 1, and in the lower part the results are summarized in columns according to the $CaCO_3$ additions.

The results for the separate groups show that the effect of the $CaCO_3$ additions was marked. Taking the diets in order of the increasing amounts of added $CaCO_3$ (columns 1–4), the numbers of young weaned were, in row 1: 20, 31, 22 and 16; in row 2: 20, 33, 10 and 10; and in row 3: 14, 27, 29 and 8. (See below for row 4, which is treated separately.) Naturally the total weights weaned depended to a large extent on the numbers weaned, so that for these three rows the total weight weaned was highest in column 2, with a marked falling off in columns 3 and 4. Considering the weight per litter weaned, thus eliminating the effect of litters dying before weaning, the same gradation is apparent in the results, the highest weight being found in column 2, with a marked falling off in columns 3 and 4. In row 4, column 2 shows a lower number weaned and total weight weaned than does column 1, owing to the loss of a complete litter, but the weight per litter weaned is as high as in column 1, and there is the same marked falling off in columns 3 and 4 as in the first three rows. Differences between the rows did not reach conventional levels of statistical significance, and the results may therefore be summarized in columns according to the $CaCO_3$ additions. This is done in the lower part of Table 2. Analysis of these results showed that at the highest level of Ca (column 4) there was a significant decrease in the total weight weaned ($P \ll 0.001$) and

in the total number weaned ($P \ll 0{\cdot}001$), and a significant increase in both the number and proportion of deaths ($P < 0{\cdot}01$). Although the differences in the numbers born at the different $CaCO_3$ levels were not statistically significant, it may be noted that there was some decrease at the two higher levels (columns 3 and 4 of Table 2, lower part). In all the measurements the best results were obtained in column 2, i.e. with a $CaCO_3$ addition of 0·5 g/100 g (or 105 g for row 4) of basal diet.

Table 2. *Exp.* 1. *Reproductive results for first litters. Each dietary group comprised four females continuously mated*

(See Table 1 for factorial design of experiment)

Statistic	Row no.	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration: 0	0·5	1·0	2·0
No. weaned/no. born	1	20/29	31/34	22/31	16/26
	2	20/30	33/33	10/29	10/18
	3	14/33	27/29	29/29	8/31
	4	28/33	24/33	12/26	5/30
No. of litters weaned/no. of litters born	1	4/4	4/4	4/4	4/4
	2	4/4	4/4	2/4	2/3*
	3	3/4	4/4	4/4	2/4
	4	4/4	3/4	2/4	2/4
Total weight weaned (g)	1	122·3	187·3	150·7	91·3
	2	157·7	237·6	84·7	56·8
	3	116·0	215·5	161·9	58·3
	4	211·0	159·2	75·8	26·2
Weight/litter weaned (g)	1	30·6	46·8	37·7	22·8
	2	39·4	59·4	42·4	28·4
	3	38·7	53·9	40·5	29·2
	4	52·8	53·1	37·9	13·1
Totals for rows 1–4:					
Born (no.)	—	125	129	115	105
Weaned (no.)	—	82	115	73	39
Deaths (no.)	—	43	14	42	66
Proportion of deaths (%)	—	34·4	10·9	36·5	62·8
Litters born (no.)	—	16	16	16	15*
Failures to rear litters (no.)	—	1	1	4	5
Total wt. weaned (g)	—	607·0	799·6	473·1	232·6
Wt./litter weaned (g)	—	40·5	53·3	39·4	23·3

* One mated female of experimental group no. 73 failed to conceive because the male was infertile.

Second and subsequent litters. Results are not given separately for the second and subsequent litters, which showed greater variability. In general, the tendency in the later litters on all diets was towards improvement, as has been found in previous work at this Institute with borderline deficiency diets (Richards, 1949*b*), but a few animals that suffered from vaginal prolapse failed to produce any litters after the first. An investigation of these cases indicated that the prolapses should be attributed to genetic rather than dietary causes. Adjustments were therefore made by assuming that the measurements for these animals were equal to the means for their dietary groups.

The complete reproductive results for all litters produced, up to four litters in a few animals, are summarized in Table 3, which gives the figures obtained in the experi-

ments, and in Table 4, which gives the treatment means adjusted for litter-mate effects and for vaginal prolapses. There was a significant decrease in the total weight of animals weaned on diets containing the highest level of $CaCO_3$ ($P=0{\cdot}001$) and in the total number of animals weaned ($P<0{\cdot}01$). The average number of deaths on diets with this level of $CaCO_3$ was significantly higher than on diets with lower levels of $CaCO_3$ ($P=0{\cdot}01$), and the highest proportion of deaths also occurred with the highest

Table 3. *Exp.* 1. *Summary of the reproductive results for the whole period of the experiment (continuous mating for* 80 *days), arranged in columns according to the* $CaCO_3$ *additions, sixteen females being tested for each level of* $CaCO_3$

	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
Statistic	0	0·5	1·0	2·0
Total young born (no.)	313	307	290	274
Total young weaned (no.)	247	274	217	154
No. of deaths:				
Total	66	33	73	120
In 1st week of life	23	14	48	53
In 2nd week of life	11	2	4	2
In 3rd week of life	32	17	21	65
Total proportion of deaths (%)	21·1	10·7	25·2	43·8
Litters born (no.)	42	37	42	42
Litters reared (no.)	38	36	35	33
Failures to rear litters (no.)	4	1	7	9
Total weight weaned (g)	2065·0	2082·8	1739·1	1144·8
Weight weaned/litter (g)	54·3	57·9	49·7	34·7

Table 4. *Exp.* 1. *Treatment means for reproductive data after adjustment for litter differences and vaginal prolapses*

	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
Mean per mated female	0	0·5	1·0	2·0
Total young born (no.)	19·6	19·2	18·2	17·1
Total young weaned (no.)	16·4	17·8	14·6	9·6
Total deaths (no.)	4·1	2·3	3·8	7·5
Total proportion of deaths (%)	0·21	0·12	0·22	0·48
Litters born (no.)	2·6	2·3	2·6	2·6
Total weight weaned (g)	138·2	135·7	116·8	71·6

$CaCO_3$ level ($P<0{\cdot}001$). The chief danger periods in the rearing of the young mice were the 1st and 3rd weeks of life, relatively few deaths occurring in the 2nd week. In both the danger periods the number of deaths in column 4 was about four times that in column 2. There was no significant effect on the total number of litters or total number of animals born.

EXPERIMENT 2

The significant effects on reproduction resulting from the $CaCO_3$ supplements used in Exp. 1 directed attention to the Ca content of the diets and the possible influence of the calcium:phosphorus ratio. From direct chemical analysis of the individual constituents the Ca and P contents of the diets and their Ca:P ratios were calculated.

The Ca contents of the diets in the four columns of Table 1, taken in order of increasing amounts of added $CaCO_3$, averaged 0·34, 0·54, 0·73 and 1·11 %, and the Ca:P ratios 0·70, 1·1, 1·5 and 2·3. The best results for most measurements were found with the diets quoted in column 2 of Table 1, i.e. with the diets whose Ca content averaged 0·54 % and whose Ca:P ratio was about 1·1, whereas in column 4, which showed the values worst in all respects, the corresponding figures were roughly twice these. Exp. 2 was therefore planned so as to test the effect of reducing the higher Ca:P ratios with a phosphorus supplement. Further, post-mortem examination of the weanlings of Exp. 1 (see Part 2 of this paper) had suggested the presence of anaemia, and this was confirmed by haemoglobin determinations (Greig, 1952). The possibility of an iron deficiency was therefore also tested in Exp. 2.

Design

In Exp. 2 the basal diet chosen was diet 78 of Exp. 1 (Table 1). The breeding mice were divided into eight groups, each containing eight animals. The groups were arranged in four pairs, one group of each pair having per 105 g basal diet a 0·5 g

Table 5. *Exp.* 2. *Plan showing the calcium and phosphorus contents and the* Ca:P *ratios of the diets*

		$CaCO_3$ (g) added to 105 g basal diet							
		0·5 (column 1)				2·0 (column 2)			
Row no.	Diet	Group no.	Ca (%)	P (%)	Ca:P	Group no.	Ca (%)	P (%)	Ca:P
1	Basal diet no. 78 Ground whole wheat 66 Casein 5 Dried whole milk 33 NaCl 1 Total 105	79	0·55	0·48	1·15	81	1·10	0·48	2·29
2	Diet no. 78 + Fe (10 p.p.m.)	89	0·55	0·48	1·15	90	1·10	0·48	2·29
3	Diet no. 78 + 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	91	0·54	1·00	0·54	92	1·07	0·98	1·09
4	Diet no. 78 + Fe (10 p.p.m.) + 2·87 g sodium dihydrogen phosphate	93	0·54	1·00	0·54	94	1·07	0·98	1·09

$CaCO_3$ supplement and the other 2·0 g (Table 5). These two levels were chosen because in Exp. 1 they had produced respectively the best and worst results. The first pair, with no addition to the basal diet except $CaCO_3$, thus received the same treatment as groups 79 and 81 of Exp. 1. For the second pair, i.e. groups 89 and 90, the iron content of the diet was increased from 35 p.p.m. (determined spectrographically) to 45 p.p.m. by adding 5·624 mg ferric citrate ($C_3H_4OH(COO)_3Fe.3H_2O$), equivalent to 1·050 mg Fe, to 105 g basal diet. For the third pair, i.e. groups 91 and 92, 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) were added per 105 g basal diet, to make the Ca:P ratio of the high-Ca group (group 92) approximately equal to that of group 79. For the fourth pair, i.e. groups 93 and 94, ferric citrate and sodium dihydrogen phosphate were added together in the same amounts as for the two

preceding pairs of groups. The plan of the experiment, showing the Ca and P contents of the diets and their Ca:P ratios, is given in Table 5.

Table 6. *Exp.* 2. *Total weight weaned and weight/litter weaned for first and second litters*

(See Table 5 for explanation of dietary groups. The nature of the dietary supplements other than Ca is indicated in parentheses below the number of the group)

Lower Ca groups				Higher Ca groups			
No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)	No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)
79	1	480·4	60·1	81	1	302·0	50·3
(nil)	2	390·3	65·1	(nil)	2	365·9	52·3
89	1	466·9	66·7	90	1	321·6	64·3
(Fe)	2	510·5	72·9	(Fe)	2	578·8	72·4
91	1	464·7	58·1	92	1	365·9	61·0
(P)	2	458·5	65·5	(P)	2	422·6	60·4
93	1	459·2	57·4	94	1	459·8	57·5
(Fe+P)	2	610·0	76·3	(Fe+P)	2	564·9	80·7

Each group contained eight females continuously mated.

Results

Number born. The addition of Fe, P or the higher level of Ca had no significant effect on the number of young born, either in the first or second litters. Combining the figures for both litters, the average numbers born per litter in the four lower Ca groups, taken in the order of column 1 in Table 5, were 8·8, 8·5, 7·1 and 9·1; and in the corresponding higher Ca groups 7·6, 9·2, 7·7 and 8·1. The total numbers born were 518 for thirty-two mice in the lower Ca groups and 491 in the higher Ca groups with one animal fewer. One mated female in group 81 failed to produce a litter within the time limit of the experiment and was therefore discarded. It was later proved that the male was infertile.

Number and weight weaned. The number weaned and total weight weaned were considerably affected by a number of failures to rear first litters. There were six of these failures among the thirty-one females in the higher Ca groups and only one among the thirty-two females in the lower Ca groups, but the mice that failed with their first litters all succeeded in rearing some of the young in their next litters, so that the total number of deaths did not eventually differ greatly in the two sets of groups, averaging 1·6 per litter born in the lower Ca groups and 1·8 in the higher Ca groups. This failure to rear first litters on the higher Ca diets, followed by an improved performance with their second litters, was also observed in another experiment (unpublished) done at the same time. In the unpublished experiment, seven of eighteen mice on diets containing the higher level of $CaCO_3$ failed to rear their first litters, but five of the seven succeeded in rearing part of their second litters. The incidence of failures to rear first litters on the higher level of Ca is not significant in the conventional statistical sense, but nevertheless appears to be of enough interest to warrant repetition of the experiment

with greater numbers. This ability to rear some young in their second litters, although they had failed to do so in first litters, is commonly observed with small laboratory animals under unfavourable dietary conditions. It may be due to the mother's power of adaptation to those dietary conditions, or possibly to her own lessened requirement for some dietary essential.

Because of the number of complete-litter failures it seems best to consider the weights weaned both as total weight weaned and weight per litter weaned. These have been collected in Table 6, in which the results for first and second litters are given separately. Considering the results for the first litters only, the influence of the complete failures is reflected in the total weights weaned. For each of the first three pairs of groups the total weight weaned in the higher Ca group (nos. 81, 90 or 92) was much below that in the corresponding lower Ca group (nos. 79, 89 or 91). For the last pair (nos. 93 *v.* 94), in which there were no complete failures, the weight weaned was the same in both groups. The diet had thus been improved by the addition of both Fe and phosphate. Considering the mean weaning weights of the first litters that were reared (Table 6), the depressing effect of the higher $CaCO_3$ level is still apparent in the first pair of groups (nos. 79 *v.* 81), but this effect has been eliminated by the addition of Fe (groups nos. 89, 90) and also by the addition of phosphate (groups nos. 91, 92) and of Fe and phosphate together (groups nos. 93, 94). For the second litters the weaning weights per litter show an increase in all groups except one, no. 92, where there is almost no change, the improvement being particularly marked in groups nos. 93 and 94, which received supplements of both Fe and phosphate.

2. EFFECTS ON ORGAN WEIGHTS

Marked differences in the weights and general condition of the young mice in the different dietary groups of Exp. 1 led to post-mortem examination of some of the weanlings when they were killed at 21 days of age. This revealed the frequent occurrence of pale speckled livers, enlarged hearts and small thymus glands, and it soon became evident that these conditions were more pronounced on the diets with the higher additions of $CaCO_3$. As an example of these findings, Pl. 1, 1 shows the marked differences in the colour of the livers between a litter of weanlings from group no. 78 (no added $CaCO_3$) and one from group no. 80 (added $CaCO_3 = 1{\cdot}0$ g/105 g of diet 78). Differences in heart and thymus weights of these two litters were also marked, as can be seen from figures given in the description of Pl. 1, 1. For comparison there are included the mean heart and thymus weights for fifteen normal litters on various stock diets outwith the present experiments.

The remaining litters of Exp. 1, which included some of the second and all subsequent litters, were therefore examined more systematically, weights being recorded for thymus, heart, liver, spleen and kidneys of representative mice from each litter.

EXPERIMENT 1

Heart and thymus weights. As the investigation of organ weights was not begun from the start of the experiment, the results are incomplete and are therefore not recorded in detail, but sufficient evidence is available to show that there was a significant

effect of $CaCO_3$ in increasing the heart weights of the weanlings. The heart weights of the mothers were not significantly affected. Although the effect of $CaCO_3$ on the thymus weights of the weanlings was not found to be statistically significant when all the results were analysed, the figures for heart and thymus weights of the groups in row 4 (Table 1), in which most litters were available, show the tendency for thymus weights to decrease as heart weights increase with increasing amounts of added $CaCO_3$. The figures are:

Organ weights (mg/100g body-weight) of animals in row 4 of Table 1

	Column 1	Column 2	Column 3	Column 4
Heart	906	959	1418	2133
Thymus	333	329	231	158

Other organs. Analysis of the weights (mg/100g body-weight) of liver, spleen and kidneys of the young mice and their mothers did not reveal any significant effects of calcium or other dietary factors on these organs.

EXPERIMENT 2

Methods

In Exp. 2, post-mortem examinations were made of weanlings from all litters. As a rule four mice (if possible two males and two females) were examined from each litter. Heart weights were recorded for both first and second litters and also for the mothers, and the hearts, together with the median lobes of the livers, were stored in 4 % (w/v) formol-saline (10 parts commercial formalin in 90 parts normal saline) for histological examination (see Greig, 1952). In view of the possibility that the heart enlargement found in these experiments might be due, in part at least, to oedema of the heart muscle, estimations were made of the dry-matter content of the hearts from second litters not required for histology. Since these had been stored in isotonic formol-saline, any effect of the fixative on the weight of dry matter would be negligible, particularly for comparative purposes.

Results

Heart weights of weanlings

Absolute heart weights. In general, the weights of organs have little significance as absolute values and must be related to body-weights, but in this experiment the absolute increases in heart weights for weanlings of the same age in the higher Ca groups were very striking. The mean heart weights and corresponding body-weights for the first litters of all groups are given in Table 7. The table shows the number of litters and also the total number of weanlings included in each group average. When the pairs of groups have almost the same mean body-weight, the effect of the higher level of Ca in increasing heart weight is clearly observed, e.g. groups nos. 79 *v.* 81, 89 *v.* 90, and 93 *v.* 94, the increase being especially marked in the first and third of these pairs of groups. In groups nos. 91 *v.* 92 the effect is even more striking, since the higher Ca group, in spite of having a lower body-weight, yet has a considerably higher heart weight. Diminution of heart weight by Fe is seen by comparing groups

nos. 81 with 90, 92 with 94, and 91 with 93. Both Ca and Fe effects were significant ($P < 0.001$).

Pl. 1, 2 illustrates this result. The mouse on the left, which may be regarded as a normal weanling, represents a litter from group 89, receiving added Fe; the heart weights and body-weights of the litter averaged 60 mg and 10 g respectively. The animal on the right came from group no. 92, the high Ca plus phosphate group; the heart weights of this litter averaged 131 mg and the body-weights 6·6 g. We have occasionally had a weanling with a heart heavier than that of its mother, although her body-weight was from five to six times as great as that of her offspring.

Table 7. *Exp.* 2. *Mean heart weights and corresponding mean body-weights of weanlings from first litters.*

(The figures in the table are group averages calculated from the litter means. The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

Lower Ca groups				Higher Ca groups			
No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*	No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*
79 (nil)	57·9	7·78	8 (34)	81 (nil)	87·4	7·82	6 (23)
89 (Fe)	68·4	9·18	7 (28)	90 (Fe)	75·3	9·17	5 (20)
91 (P)	72·5	9·36	8 (34)	92 (P)	85·9	8·79	6 (26)
93 (Fe+P)	51·8	8·08	8 (28)	94 (Fe+P)	69·9	8·15	8 (30)

* Figure in parentheses gives the number of young.

Table 8. *Exp.* 2. *Mean relative heart weights of weanlings (first and second litters) and mothers; and mean relative thymus weights of weanlings (second litters)*

(The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

	Mean heart weight (mg/100 g body-weight)*			Mean thymus weight (mg/100 g body-weight) second litters*
No. of group	First litters	Second litters	Mothers	
		Lower Ca groups		
79 (nil)	754 (8)	790 (6)	492 (8)	358 (6)
89 (Fe)	745 (7)	636 (7)	487 (8)	423 (7)
91 (P)	784 (8)	690 (7)	450 (8)	399 (7)
93 (Fe+P)	645 (8)	621 (8)	462 (8)	439 (8)
		Higher Ca groups		
81 (nil)	1154 (6)	1022 (6)	524 (7)	283 (6)
90 (Fe)	843 (5)	783 (5)	484 (8)	323 (8)
92 (P)	993 (6)	954 (5)	531 (8)	275 (7)
94 (Fe+P)	871 (8)	753 (7)	530 (8)	401 (7)

* Figure in parentheses gives the number of litters or mothers included in the mean.

Relative heart weights. The effects of the dietary supplements on the ratio of heart weight to body-weight are shown in Table 8. By comparing the values for each pair of diets (groups nos. 79 *v.* 81, 89 *v.* 90, 91 *v.* 92, and 93 *v.* 94) it is seen that the higher level of Ca increased the relative heart weight. This effect is significant ($P < 0.001$). The effect of Fe in decreasing relative heart weight, both at the lower and the higher

Ca level, is also significant ($P<0{\cdot}001$), and is evident from a comparison of the relevant groups (nos. 79 *v.* 89, 91 *v.* 93, 81 *v.* 90, and 92 *v.* 94). The effect of the phosphate addition on relative heart weight was not statistically significant. Groups nos. 92 and 93, with added phosphate, showed both in first and second litters slight reductions in relative heart weight compared with groups nos. 81 and 89, the corresponding

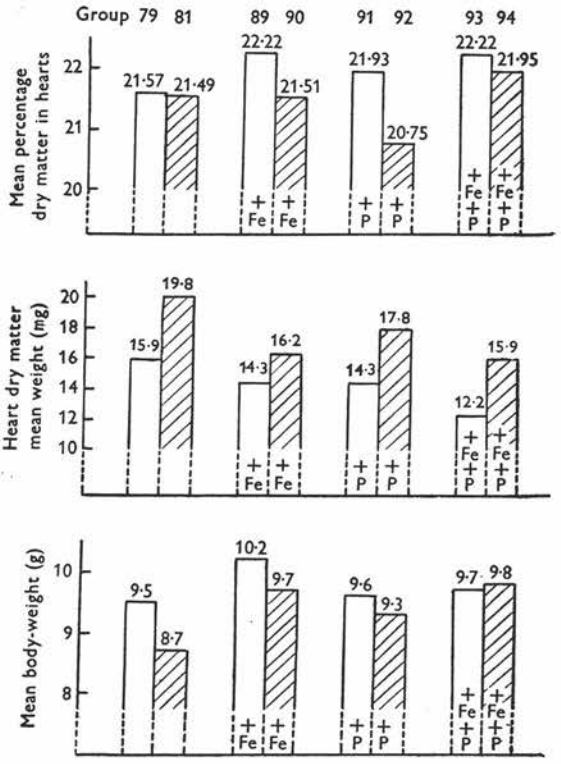


Fig. 1. Exp. 2. Diagram showing mean percentage dry matter in hearts, mean absolute weight of heart dry matter and mean body-weight of second litters. The weight of heart dry matter is greater in the higher Ca group of each pair (shaded columns), in spite of decreased percentage dry matter in heart and diminished body-weight in most animals. For dietary groups see Table 5.

groups without phosphate; but when groups nos. 91 and 94, with added phosphate, are compared with the corresponding groups nos. 79 and 90, the reduction is seen only in the second litters. It may be noted that in all groups except group no. 79 the relative heart weights of second litters were less than those of first litters, that is, they showed an improvement.

Heart dry matter. The percentage of dry matter in the heart was significantly lowered by the higher level of Ca ($P<0{\cdot}001$) and significantly increased by Fe ($P<0{\cdot}001$), whereas phosphate had no significant effect (Fig. 1). Examination of the results showed, however, that the increased moisture content of the hearts in the higher Ca groups was by no means sufficient to account for the increased weights observed. The weights of dry matter present in the hearts, along with the percentage dry-matter contents and the average body-weights of the different groups, are shown

diagrammatically in Fig. 1. The increased absolute weight of dry matter in the hearts of the higher Ca groups is clear.

Relation between heart weights and blood haemoglobin. We have found that a highly significant correlation exists between the heart enlargement in the young mice and the degree of anaemia, as shown by the blood-haemoglobin estimations described by Greig (1952). The closeness of this relationship can be seen from the scatter diagram

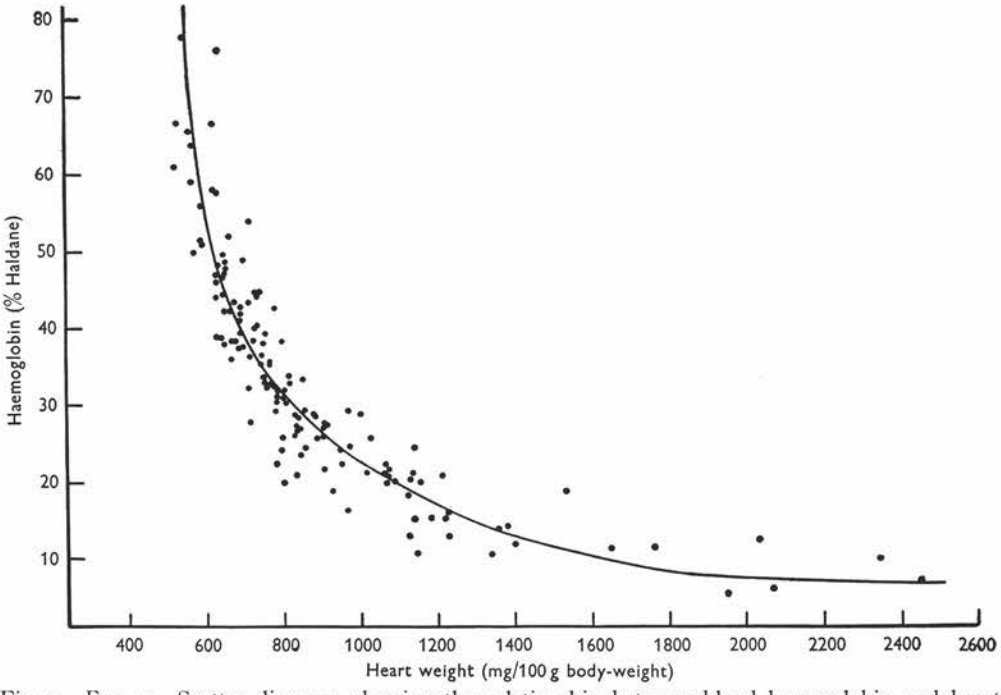


Fig. 2. Exp. 2. Scatter diagram, showing the relationship between blood haemoglobin and heart weight relative to body-weight, for 141 litters irrespective of diet. Each point represents the mean value for one litter.

in Fig. 2, in which Hb is plotted against heart weight expressed in mg/100 g body-weight (H.W. %) for all litters in Exp. 2, irrespective of their group, and for thirty-three litters from the unpublished experiment mentioned in part 1 of this paper. Statistical analysis showed that the relationship between log H.W. % and log Hb was inverse and linear. For the 141 litters observed the correlation coefficient r was $-0{\cdot}936 \pm 0{\cdot}010$, the regression equations for the prediction of one variable from the other being:

$$\log \text{H.W. } \% = 3{\cdot}780 - 0{\cdot}580 \log \text{Hb} \quad \pm 0{\cdot}018,$$

and

$$\log \text{Hb} = 5{\cdot}955 - 1{\cdot}533 \log \text{H.W. } \% \pm 0{\cdot}049,$$

where Hb is expressed as a percentage of the Haldane standard (100 % = 14·8 g haemoglobin/100 ml. blood), and H.W. % = mg heart/100 g body-weight.

Heart weights of mothers

The heart weights of the mothers were also affected by the Ca level of the diet. The higher level of Ca increased absolute heart weight ($P < 0{\cdot}05$), increased heart

weight relative to body-weight ($P<0{\cdot}001$) and decreased the percentage of heart dry matter ($P<0{\cdot}05$). The mean relative heart weights are shown in Table 8. Comparison of the groups shows that the addition of Fe or phosphate did not cause a statistically significant reduction in the heart weights of the mothers. It is possible that the effects would have been more pronounced had the animals been reared on the experimental diets from weaning.

Thymus weights

Thymus weights of second litters were significantly affected both by the Ca and Fe additions, but in the opposite direction from the heart weights. Thus the absolute weight of the thymus and its weight relative to body-weight were both diminished by the higher level of Ca ($P<0{\cdot}001$) and increased by the addition of Fe ($P<0{\cdot}001$). Again the addition of P had no significant effect. The mean weights of the thymus relative to body-weight are shown in Table 8. The figures show clearly both the diminution in weight in the higher Ca groups and the increase in the groups receiving Fe.

DISCUSSION

In these mouse experiments the adverse effects of $CaCO_3$ on reproduction were seen mainly in a reduction of the numbers weaned, caused by complete or partial failure to rear litters, and in a reduction of the weaning weights of the surviving litters. The number of deaths in the 1st week of life was high. Doubtless some of these deaths, in all groups, must be attributed to causes other than diet, since deaths in the first few days of life are not unusual with small laboratory animals even on normal diets; it is also possible that with the system of continuous monogamous mating the presence of the male may have been an additional disturbing factor. The figures given for Exp. 1 indicate, however, that diet must have been the major cause of these early deaths, since the mortality on the two higher levels of $CaCO_3$ was much greater than on the two lower levels. In the 3rd week the mortality was greatest on the highest level of $CaCO_3$, and it seems justifiable to attribute almost all the deaths during this period to dietary causes. The mother's milk may have been deficient in quality or quantity, or possibly some defect in the young animal may have prevented its proper utilization. This could account both for the large number of deaths during this period and for the decreased weaning weights of the survivors. The most likely explanation of these findings would appear to be an induced Fe deficiency in the mothers, which may have led to a reduced Fe content of the milk, always very low, or to a reduced iron reserve in the young animal at birth.

Although the adverse effects of the $CaCO_3$ supplements in Exp. 1 were more apparent on the rearing of the young than on the fertility or fecundity of the mothers, there was, nevertheless, a tendency towards decrease in the numbers born at the two higher levels. The number of animals in each group of that experiment was small, and it seems possible that with larger numbers the decrease in the birth rate might have reached significance. In this connexion it may be noted that in the experiments of Howie & Porter (1950), with twenty-two breeding females in each group, the animals

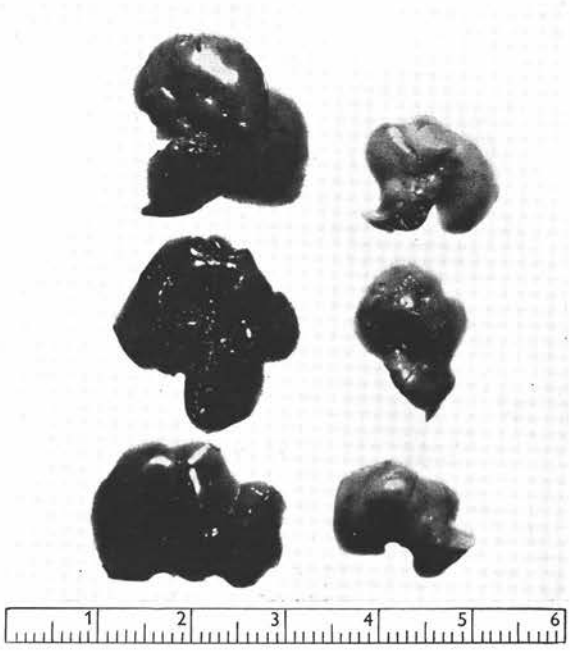
on their diet 2 were not only markedly superior in the number and weight of young weaned to those on their diet 5, but also showed a marked increase in the number of young born. One of the main differences between these two diets was that diet 5 contained 1 % of added $CaCO_3$. The differences in our experiments could perhaps also have been increased by giving the experimental diets to the breeding females at weaning instead of at 7 weeks of age.

As to the mode of action of $CaCO_3$ in impairing reproduction, our results suggest that a dietary Ca:Fe relationship was involved. Excess of Ca seemed to reduce in some way the amount of available Fe, and improvement was effected by increasing the proportion of Fe to Ca, as was shown by the improved weaning weights of the Fe groups, particularly in the second litters. This point is more fully discussed in the second paper of this series (Greig, 1952). To what extent the Ca:P ratio was involved is less clear. Reduction of this ratio by the addition of phosphate did effect some improvement in the average weaning weights of the litters, but to a smaller extent than did the addition of Fe. The effect of P on the organ weights of the weanlings was also much less marked than that of Fe. Thus our results indicate that the Ca:Fe relationship was more important than the Ca:P ratio.

The reproductive findings in these experiments are of interest in view of a recent paper by Hignett (1950), who considers that calcium-phosphorus imbalance in the diet is one cause of herd infertility in cattle. He contends that the $CaO:P_2O_5$ ratio should not be much wider than 1:1, with a sufficient intake of P, and mentions instances of herds in which breeding efficiency was markedly improved by a narrowing of the Ca:P ratio. Shaw (1950) also reported infertility in cows in a district with a hard-water supply, where heavy liming was practised, and stated that a general improvement in the conception rate corresponded to decreased liming.

Our experiments also showed that hypertrophy of the heart and atrophy of the thymus gland were produced in weanling mice by additions of $CaCO_3$ to their mothers' diet. Similar changes were reported by Schmidt (1928) in young mice on a low-iron diet of milk and rice and suffering from a direct iron-deficiency anaemia. He found that the abnormalities could be completely prevented by administering iron in the diet. Since in our experiments the heart and thymus changes could also be prevented by supplementing the mothers' diet with Fe, there seems little doubt that they were associated with an iron deficiency, produced indirectly by the $CaCO_3$ additions to the diet. Thus the heart hypertrophy and thymus atrophy would seem to result from the same essential cause as the impairment in reproduction, namely an imbalance in the dietary Ca:Fe relationship.

The finding that the principal cause of the increase in heart size was an increase in its dry-matter content indicates that a compensatory hypertrophy of the heart muscle resulted from the anaemia. The atrophy of the thymus may have been a consequence of the general under-development frequently observed in the young mice. It is conceivable, however, that the cause of the atrophy may have been purely physical, and that 'accidental involution' resulted from compression of the gland by the enlarged heart. On the other hand, it is known that pyridoxin deficiency causes anæmia in dogs and swine, that it leads to thymus atrophy in young rats and that the effects of



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the deficiency are intensified by additions of $CaCO_3$ to the diet. There may therefore be some more fundamental relationship between the thymus atrophy and the anaemia induced by the $CaCO_3$ additions to the diet.

SUMMARY

1. In a breeding test on mice, using the method of continuous monogamous mating, three levels of calcium carbonate were added to a slightly modified B diet of Sherman and to three further modifications of it. On all the diets the highest addition of calcium carbonate, which gave a Ca intake of 1·1 % and a Ca:P ratio of approximately 2·3, significantly lowered the number and total weight of young weaned and increased the number and proportion of deaths. There was also some decrease in the number of births.

2. When supplements of ferric citrate and sodium dihydrogen phosphate were added to the diet, a number of mothers in the high Ca groups failed to rear their first litters, but in the litters that were reared the weaning weights seemed to be favourably affected by the addition of Fe or by a reduction of the Ca:P ratio.

3. Both absolutely and relatively to body-weight the heart weights of the weanlings were significantly increased by Ca and decreased by Fe, whereas thymus weights were decreased by Ca and increased by Fe. The high Ca level also caused a significant increase in the absolute and relative heart weights of the mothers.

4. Although the percentage of dry matter in the hearts of the weanlings was significantly lowered by the higher level of Ca, there was a marked increase in the absolute weight of heart dry matter at this level, indicating the presence of a cardiac hypertrophy.

5. A highly significant relationship was found between heart weight and blood haemoglobin in the weanling mice, an increase in heart weight accompanying a decrease in Hb. The relationship between log heart weight, expressed in mg/100 g body-weight, and log Hb was linear.

We are indebted to Mr H. M. Quenouille, Statistics Department, University of Aberdeen, for statistical analysis of our data, to Dr R. L. Mitchell, Macaula Institute for Soil Research, Aberdeen, for spectrographic determinations of iron in the foodstuffs, and to Mr J. Davidson, of this Institute, for their chemical analysis. We wish also to thank Miss Isabel Knowles for assistance in the care and feeding of the animals.

EXPLANATION OF PLATE

1. Exp. 1. Livers of weanling mice showing the effect on liver colour of adding $CaCO_3$ to the mother's diet. (Scale in cm.) Left, livers from group no. 78 (no added $CaCO_3$); right, livers from group no. 80 (1·0 g $CaCO_3$ added to 105 g of diet 78). Mean heart and thymus weights (mg/100 g body-weight) for these two litters and for fifteen normal litters on various stock diets were:

	Heart	Thymus
Litter from group no. 78	952	310
Litter from group no. 80	1987	179
Normal litters	645	471

2. Exp. 2. The weanling mouse on the left from group no. 89, receiving added Fe, shows normal heart, thymus and liver. That on the right from group no. 92 shows the enlarged heart, small thymus and pale liver characteristic of weanlings from mothers receiving excess of $CaCO_3$ in the diet. (Scale in cm.)

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The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

2. Haematology and Histopathology

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Richards & Greig (1952) reported pale livers and enlarged flabby hearts in weanling mice whose mothers' diets contained 1–2 % of added calcium carbonate. It seemed likely that the calcium carbonate had induced an unexpected anaemia in the young.

The work reported in the present paper was undertaken to investigate this possibility and to examine the pathology of the condition. The presence of anaemia was confirmed by haemoglobin estimations, and fuller examination of the blood indicated that it was probably of the iron-deficiency type. A further experiment showed that the anaemia could be prevented by feeding an iron supplement, but not by balancing the additional dietary calcium with sufficient phosphorus to restore the original Ca:P ratio of the diet.

METHODS

The two reproduction experiments reported by Richards & Greig (1952) provided the material for this work. The designs of both experiments have already been described in detail by these authors, and need only brief mention here.

Experiment 1 (Richards & Greig, 1952).

Design. Sixteen litters of four female mice, stratified by weight, were allocated to four Latin squares with litter-mates distributed orthogonally. The other component variables of the squares were the calcium carbonate level of the diet (in vertical columns), and the levels of wheat and of casein in the diet factor (in horizontal rows).

Each diet was a modification of the B diet of Sherman (Sherman & Campbell, 1924). Continuous monogamous mating was allowed for 80 days.

Haematological. All the weanlings in each available litter were examined at 21 days of age. The axillary vessels were resected under ether anaesthesia, and a sample of blood was withdrawn in a Haldane haemoglobin pipette. For a fuller blood examination, approximately 0·5 ml. was withdrawn and heparinized. A drop of fresh blood was used to prepare a blood film, and the animal was forthwith destroyed with ether. All subsequent examinations were performed on the heparinized samples. The mothers were dealt with in the same way, as they became available at the end of the breeding experiment.

Haemoglobin (Hb) was estimated by Haldane's (1901) carboxyhaemoglobin method. Erythrocyte counts (R.B.C.) and leucocyte counts (W.B.C.) were performed in the usual way, using Thoma pipettes and Neubauer haemocytometers, with Hayem's fluid and 1 % (v/v) acetic acid as diluents. Packed erythrocyte volumes (P.C.V.) were determined by means of capillary microhaematocrit tubes, spun for 1 h at 3000 r.p.m. (R.C.F. = *c.* 1500 *g*). Blood films were stained with Leishman's stain. The same items of apparatus were used throughout the observations, which were made by the same observers. The mean corpuscular haemoglobin (M.C.H.), mean corpuscular haemoglobin concentration (M.C.H.C.) and mean cell volume (M.C.V.) were then calculated from the appropriate observed data.

Histological. The hearts, livers, kidneys, spleens and thymus glands of both litters and mothers were fixed in 4 % (w/v) formol-saline (10 parts commercial formalin in 90 parts normal saline), or in formol-corrosive (10 parts commercial formalin in 90 parts saturated aqueous solution of mercuric chloride) (Lendrum, 1943). Paraffin sections were prepared in the usual manner, and stained by Mayer's (1903–4) haemalum and eosin. Frozen sections, stained with Scharlach R and haemalum, were prepared from the median lobe of the liver of each mother and of a representative of each litter. Smears were made from the femoral bone marrow of a number of weanlings and mothers in various diet-groups and stained with Leishman's stain. In addition, sections of bone marrow were prepared by the method advocated for sternal puncture material by Cappell, Hutchison and Smith (1947); these were stained with haemalum and eosin.

Experiment 2 (Richards & Greig, 1952)

Design. Eight replicates of a 2^3 factorial design were employed, the dietary variables being low or high levels of $CaCO_3$ (0·5 or 2·0 g, respectively, added to 105 g basal diet), and the presence or absence of supplementary iron (10 p.p.m. as ferric citrate) or phosphorus (0·57 % as $NaH_2PO_4.2H_2O$) or both. The sixty-four female mice, in litters of four, were distributed so that two litters were allotted to each block of eight dietary treatments, with the interactions $CaCO_3 \times Fe$, $CaCO_3 \times P$, $Fe \times P$ and $CaCO_3 \times Fe \times P$ partially confounded between litters. Each mother was allowed to become pregnant for the second time before the male was removed from the cage.

Haematological. Each litter was represented, where possible, by two males and two females; these were the two animals nearest to the median weight of the weanlings

of each sex in the litter. In general the same procedure was followed as in Exp. 1, but the blood picture of all first litters and mothers was examined. Heller & Paul's (1934) oxalate anti-coagulant mixture was used in place of heparin; leucocyte counts were omitted, but reticulocytes were examined in blood films stained supravitally with brilliant cresyl blue and counterstained with Leishman's stain. Only Hb estimations were done on the whole blood of second litters, but pooled samples of serum from each second litter were used to estimate serum inorganic phosphorus by King's (1946) method.

Histological. Frozen sections of the liver of each mother and of a representative of each first litter were examined.

RESULTS

Haematology

Experiment 1

Litters. Since the haematological observations were begun only towards the end of the experiment, the data were incomplete. No first litters and very few second litters were available, so that most of the observations were on third and fourth litters. Diet-group means were computed by taking the mean for all the young in each litter,

Table 1. *Exp.* 1 (*litters*). *Mean blood haemoglobin of weanling mice from mothers with four levels of dietary calcium carbonate*

	Percentage of $CaCO_3$ added to mothers' diet*				
	0	0·5	1	2	S.E.
Hb (g/100 ml. blood)	6·96	5·77	4·88	3·85	±0·755

The values at the two lowest levels of $CaCO_3$ are significantly greater than that at the highest level ($P < 0{\cdot}01$ and $< 0{\cdot}05$ respectively).

* In four of the sixteen diets used, additions of $CaCO_3$ were per 105 parts instead of 100 parts basal diet (see Richards & Greig, 1952).

and finding from these the mean for all the litters in each diet-group. The means were not weighted for either number in litter or number of litters, and data from second, third and fourth litters were considered together. Statistical analysis of the figures was possible only for Hb; even here the small numbers precluded consideration of each diet-group separately, but the overall effects of modifications in the wheat-casein factor and of changes in the dietary $CaCO_3$ level could be determined by combining, as marginal means, the values in each row (i.e. horizontally) and at each level of $CaCO_3$ (i.e. vertically). The differences between the rows failed, in every instance, to reach significance, but the values for the first (no added $CaCO_3$) and second (0·5 % added $CaCO_3$) levels were significantly greater than the value for the last (2 % $CaCO_3$) level ($P < 0{\cdot}01$ and $< 0{\cdot}05$, respectively), whereas values for the third level were intermediate (Table 1).

Data on the other attributes are not presented here as they were too limited for a conclusive finding, but in general all measurements, including the calculated M.C.H.

and M.C.H.C. but not, however, the M.C.V., tended to fall in the last two columns, thereby suggesting that the anaemia was hypochromic. This suggestion was borne out by the appearance of the erythrocytes in the stained films. Apart from the polychromatic cells normally found in mouse blood, the red cells of anaemic weanlings were mainly hypochromic, but orthochromatic cells similar to those described by Price-Jones (1932) were also present and stood out in contrast. Anisocytosis was invariable, and poikilocytosis frequent (Pl. 1, 1*a*)

Mothers. Mean values and standard errors were calculated for each diet-group, row and $CaCO_3$ level, as was done with the litter data. All but six of the sixty-four mothers were available for Hb, R.B.C. and W.B.C. determinations. For these attributes, and for the M.C.H., the arrangement of litter-mates in the design made it possible to isolate and eliminate the component of variability attributable to litter (i.e. genetic) differences. However, P.C.V. estimations could be made on only forty-two animals, and for this attribute and for the erythrocytic indices depending on it, the design had to be treated simply as randomized blocks.

The adjusted results are summarized in Table 2. It will be seen that none of the differences between the rows reached significance; there was not even evidence of consistency between measurements. On the other hand, comparison between the

Table 2. *Exp.* 1 (*mothers*). *Blood picture of mother mice on four basal diets, each with four levels of added calcium carbonate*

(*a*) *Method of calculating marginal means for Hb* (g/100 *ml.*) *from diet-group means*

Basal diet†	Percentage of $CaCO_3$ added to diet* 0	0·5	1	2	Marginal mean
Row 1	14·19	14·61	13·85	12·71	13·84
Row 2	15·33	12·70	13·68	12·96	13·66
Row 3	15·21	14·33	15·16	12·99	14·43
Row 4	15·36	14·49	13·36	11·84	13·76
Marginal mean	15·02	14·03	14·02	12·62	13·93

S.E. of differences between Marginal means ±0·662
Diet-group means ±1·146

(*b*) *Marginal means for rows, and calcium carbonate levels determined from diet-group means as in* (*a*) *above*

Attribute	Row 1	2	3	4	Percentage of $CaCO_3$ added to diet* 0	0·5	1	2	S.E.
Hb (g/100 ml.)	13·84	13·66	14·43	13·76	15·02	14·03	14·02	12·62	±0·662
R.B.C. (10^6/cu.mm)	10·6	10·4	11·5	10·4	11·2	10·3	11·0	10·4	±0·62
P.C.V. (%)	45	41	45	42	48	40	44	42	±3·0
M.C.H. ($\mu\mu$g)	13·2	13·3	12·7	13·3	13·9	13·9	12·9	11·8	±0·92
M.C.H.C. (%)	30·7	34·1	32·3	36·8	35·8	35·8	33·7	29·1	±3·69
M.C.V. (cu.μ)	42·2	40·9	40·2	40·0	43·1	38·8	40·4	40·6	±3·37
W.B.C. (10^3/cu.mm)	3·9	2·4	2·8	2·4	2·4	2·9	2·4	3·7	±3·73

Significant differences:
Hb is lower at 2 % $CaCO_3$ level than at 0, 0·5 and 1 % levels ($P<0·01$, $<0·05$ and $<0·05$).
M.C.H. is lower at 2 % $CaCO_3$ level than at 0 and 0·5 % levels ($P<0·05$ and $<0·05$).

* In Row 4, additions of $CaCO_3$ were per 105 parts instead of 100 parts basal diet.
† For details of the composition of these diets see Richards & Greig (1952).

columns (i.e. levels of $CaCO_3$) showed that the mean Hb of animals on the diets containing the highest level of $CaCO_3$ was significantly lower than that obtained at all other levels ($P<0{\cdot}01$, $<0{\cdot}05$, $<0{\cdot}05$). The mean M.C.H. at the 2 % $CaCO_3$ level was also significantly lower than at the 0 and 0·5 % $CaCO_3$ levels ($P<0{\cdot}05$, $<0{\cdot}05$). There was also a suggestion that a similar relationship existed between the M.C.H.C. and the $CaCO_3$ level of the diet. No significant effects were apparent on the other blood attributes.

There was thus considerable evidence that, in the mothers as well as the weanlings, the anaemia was hypochromic. Examination of stained blood films bore this out; the erythrocytes in the more anaemic bloods were pale and presented the same general characters as those of the weanlings.

For further confirmation of these findings, correlation coefficients between Hb and all other attributes were calculated from the data of each individual animal, ignoring treatment effects (Table 3). From these it was clear that Hb was strongly correlated

Table 3. *Exp.* 1 (*mothers*). *Correlation coefficients between haemoglobin and other blood attributes, ignoring treatment effects*

Correlation	r	P
Hb and R.B.C.	+0·460	<0·001
Hb and P.C.V.	+0·667	<0·001
Hb and M.C.H.	+0·531	<0·001
Hb and M.C.H.C.	+0·362	<0·05
Hb and M.C.V.	+0·254	>0·10
Hb and W.B.C.	+0·313	<0·05

with M.C.H., the relationship being almost linear. This showed that in anaemic animals the haemoglobin carried by each erythrocyte diminished in proportion to the anaemia. The coefficients also confirmed that the blood of more anaemic animals (i.e. those with lower Hb values) had a lower M.C.H.C.; that is to say, the anaemia was hypochromic. No significant correlation, however, could be shown between Hb and M.C.V.; that is to say, the anaemia could not be shown to be other than normocytic. Table 3 also shows that there was a significant correlation between Hb and W.B.C., indicating that the anaemia was accompanied by a leucopenia. Except for the lack of demonstrable change in the M.C.V. (discussed later in this paper) these findings all suggested that the anaemia was of the type associated with iron deficiency.

Experiment 2

First litters. The summarized results for all haematological attributes measured are shown in Table 4(*a*). The mean value for each litter was first determined, and then the mean of all such litter means in each diet-group. These diet-group means are the figures quoted in the table.

The effects of the various treatments are in most cases obvious from inspection of the data, e.g. the effect of the higher Ca level on the unsupplemented diet is to reduce Hb from 5·00 to 2·66 g/100 ml., and to reduce R.B.C. from 5·4 to 3·4 million/cu.mm, and so forth. The significance of these differences (2·34 and 2·0 respectively) can be determined in each case by using the standard error applicable to comparisons between

Table 4. *Exp.* 2 (*first litters and mothers*). *Diet-group means and standard errors for haematological attributes, and the separate effects of the dietary supplements*

		Hb (g/100 ml.)		R.B.C. (10^6/cu.mm)		P.C.V. (%)		M.C.H. ($\mu\mu$g)		M.C.H.C. (%)		M.C.V. (cu.μ)	
	Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
a) First litters (eight litters in each diet-group)	—	5·00	2·66	5·4	3·4	21·5	13·7	9·4	7·6	23·0	18·2	41·2	41·4
	Fe	5·93	4·45	5·9	5·2	24·5	20·4	10·0	8·5	24·0	21·5	41·6	39·9
	P	4·71	3·39	5·5	4·3	20·8	16·4	8·6	7·7	22·5	19·9	38·2	38·6
	Fe+P	6·87	4·00	6·5	4·3	27·4	18·6	10·6	9·1	25·1	20·8	42·6	43·8
	S.E. of difference between means of:												
	Thirty-two litters	±0·31		±0·25		±1·05		±0·40		±0·79		±1·13	
	Sixteen litters	±0·44		±0·36		±1·48		±0·57		±1·11		±1·60	
	Eight litters	±0·63		±0·50		±2·10		±0·80		±1·57		±2·27	
	Effect of:												
	$CaCO_3$‡	−2·00***		−1·53***		−6·26***		−1·44***		−3·57***		−0·03	
	Fe‡	+1·37***		+0·83**		+4·63***		+1·24**		+1·95*		+2·09	
	P‡	+0·22		+0·18		+0·78		+0·07		+0·40		−0·22	
(*b*) Mothers (eight animals in each diet-group)	—	11·93	9·83	9·7	8·5	42·6	37·5	12·4	11·4	28·0	25·7	44·2	44·4
	Fe	14·12	12·70	10·6	10·0	51·6	43·6	13·5	12·7	27·6	28·8	48·9	43·5
	P	14·83	10·74	10·5	9·9	51·1	40·2	14·4	10·8	29·2	26·7	49·0	40·4
	Fe+P	14·43	12·79	10·2	9·4	47·9	44·9	14·1	13·8	30·2	28·6	46·9	48·1
	S.E. of difference between means of:												
	Thirty-two animals	±0·52		±0·39		±1·7		±0·44		±0·55		±1·1	
	Sixteen animals	±0·74		±0·55		±2·4		±0·62		±0·78		±1·6	
	Eight animals	±1·04		±0·78		±3·4		±0·88		±1·10		±2·2	
	Effect of:												
	$CaCO_3$‡	−2·32***		−0·82*		−6·8***		−1·45**		−1·28*		−3·2**	
	Fe‡	+1·67**		+0·42		+4·1*		+1·28**		+1·39*		+2·3*	
	P‡	+1·07*		+0·32		+2·2		+0·77		+1·09		+0·8	

† Supplements per 105 g basal diet: low $CaCO_3$, 0·5 g; high $CaCO_3$, 2·0 g; Fe, 1·05 mg (i.e. 10 p.p.m.); P, 0·57 g as $NaH_2PO_4.2H_2O$. The high $CaCO_3$+P groups and the low $CaCO_3$ without P groups had the same Ca:P ratio, namely 1·0. Basal diet: ground whole wheat 66, dried whole milk 33, casein 5, sodium chloride 1. For full details see Richards & Greig (1952).

‡ In each case means of thirty-two litters or mothers are being compared. Effects were calculated from litter means and are correct to two decimal places, although the diet-group means have been rounded off correct to the first decimal place. Significant effects are indicated thus: *** ($P<0·001$), ** ($P<0·01$) and * ($P<0·05$).

two diet-group means, i.e. the means of two sets of eight litters each. For Hb and R.B.C. these are $\pm 0{\cdot}63$ and $\pm 0{\cdot}50$; thus both differences are highly significant since they are three or four times as large as their respective standard errors. The significance of differences between any two treatments can be determined in this way.

Another estimate of the effect of a dietary treatment can be made by averaging its effects over all other treatments. This estimate is more accurate because it is based on a larger number of observations, and it is valid provided there are no important interactions. For instance, the overall effect of Fe on Hb is

$$\tfrac{1}{4}(5{\cdot}93+4{\cdot}45+6{\cdot}87+4{\cdot}00)-\tfrac{1}{4}(5{\cdot}00+2{\cdot}66+4{\cdot}71+3{\cdot}39)=1{\cdot}37.$$

This difference is more than four times its standard error (i.e. the S.E. of difference between means of sets of thirty-two litters, namely $\pm 0{\cdot}31$) and the difference is therefore highly significant ($P<0{\cdot}001$). Comparisons between means of sixteen litters can be made on the same principle. When the overall effect of the higher $CaCO_3$ level on Hb is determined in this way, it is seen to be more than six times its S.E. ($2{\cdot}00>6\times 0{\cdot}31$). The size, direction and significance of such effects are set out below each attribute in Table 4. The interactions $CaCO_3\times Fe$, $CaCO_3\times P$, $Fe\times P$ and $CaCO_3\times Fe\times P$ were all tested, and in some instances appeared to reach significance at the 5 % level; but as they were small in comparison with the main effects, and inconsistent in their directions, they could safely be ignored in considering main effects.

As can be seen from Table 4(*a*), the overall effect of $CaCO_3$ in reducing Hb, R.B.C., P.C.V., M.C.H. and M.C.H.C. was highly significant. Fe supplementation of the diet also produced significant effects on all these attributes, but in the opposite direction. In strong contrast was the fact that none of the dietary treatments could be shown to produce a significant effect on M.C.V. The effects of P never approached significance.

On this evidence, then, the higher level of $CaCO_3$ in the mother's diet induced a hypochromic, normocytic anaemia, and litters from the non-Fe-supplemented diet-groups also exhibited hypochromic anaemia when compared with the Fe-supplemented groups.

Examination of the supravitally stained smears (Pl. 1, 1*b*) showed that reticulocytes and late normoblasts were numerous in all specimens. Although systematic differential counts were not made on all smears, these cells appeared to be rather more numerous in the blood of weanlings from the high dietary-$CaCO_3$ groups. In these animals, early normoblasts were also encountered, sometimes in considerable numbers. The mature erythrocytes were hypochromic, and anisocytosis and poikilocytosis were common.

Mothers. The same procedure was followed with the data from the mothers; the results are shown in Table 4(*b*). The differences were not always so large, but the directions of the effects were similar. Here, however, the effect of P on Hb was positive and just reached significance ($P<0{\cdot}05$), and the M.C.V. was significantly reduced by $CaCO_3$ ($P<0{\cdot}01$) and increased by Fe ($P<0{\cdot}05$). The anaemia in the mothers induced by dietary $CaCO_3$ was therefore hypochromic and microcytic, whereas lack of an Fe supplement in the diet also was responsible for a relative anaemia of this type.

The stained blood smears of the mothers presented the same picture as those of the weanlings, except that young normoblasts were unusual.

Second litters. Results for second litters are shown in Table 5. The $CaCO_3$ and Fe effects on Hb were again very strong ($P < 0{\cdot}001$), and this time the effect of P was also significant ($P < 0{\cdot}05$). The figures show that, compared with first litters (Table 4*a*), second litters were improved in every diet-group ($P < 0{\cdot}01$).

Table 5. *Exp.* 2 (*second litters*). *Diet-group means and standard errors for blood haemoglobin and serum inorganic phosphorus, and the separate effects of the dietary supplements*

	Hb (g/100 ml.)		Serum inorganic P (mg/100 ml.)	
Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
—	5·77	3·12	11·8	11·5
Fe	7·76	4·53	10·8	11·0
P	6·57	3·70	12·1	10·8
Fe+P	8·52	5·98	12·9	11·2
S.E. of difference between means of:				
Thirty-two litters	±0·40		±0·44	
Sixteen litters	±0·56		±0·62	
Eight litters	±0·80		±0·87	
Effects† of:				
$CaCO_3$	−2·83***		−0·77	
Fe	+1·91***		−0·08	
P	+0·90*		+0·48	

† See footnotes to Table 4.

There were no significant effects on serum inorganic phosphorus. However, a striking hyperlipaemia was observed in serums from the most anaemic litters. This observation is receiving further study.

Histopathology

Experiment 1

Hearts. The hearts both of mothers and of litters from the higher $CaCO_3$-level groups were enlarged (Richards & Greig, 1952). They exhibited marked hypertrophy of the individual muscle fibres and oedematous separation of the muscle bundles. In a number of the most anaemic weanlings from these groups, degenerative changes were apparent in the heart-muscle cells (Pl. 1, 2). Fatty degeneration was never seen, but, in one particularly anaemic weanling, numerous calcified areas were observed scattered throughout the muscle tissue.

Thymus glands. Richards & Greig (1952) found that the thymus glands of litters from the high $CaCO_3$ diet-groups were smaller than those from other diet-groups, but histologically there were no signs of sclerosis or of degeneration in the thymocytes. However, the line of demarcation between cortex and medulla tended to be less distinct, which suggested that the organ had undergone premature involution. No differences were found in the thymus glands of mothers.

Kidneys. The only abnormal features were early degenerative changes in the tubule cells of a few of the most anaemic weanlings. There was no evidence of fatty degeneration.

Spleens. There were no important differences in the structure of the spleens of either litters or mothers on different dietary treatments.

Livers. A certain amount of visible fat was present in sections of the livers of both adult and young mice on even the best diets in this experiment. In a few specimens the quantity of fat was small (Pl. 2, 1), in many it was considerable (Pl. 2, 2), and in some enormous (Pl. 2, 3).

In the weanlings, the impression was gained that the degree of fatty change was greatest when the level of $CaCO_3$ in the mother's diet was highest. In order to corroborate this, the amount of visible fat in comparable sections from a mother and a weanling in each diet-group was assessed by an arbitrary +, ++, +++ system. With only one observation from each diet-group, the results were not suitable for statistical analysis. Nevertheless, they confirmed the impression, since in the weanlings the highest number of '+'s' was recorded from the highest $CaCO_3$ diet-group, and the lowest number from the two groups with the lowest $CaCO_3$ levels. No association was apparent between the basal diet of each row and the amount of fat present in the liver.

The livers of the mothers were much more variable in their fat content, and no general correlation between liver fat and diet was evident. It was nevertheless true that those livers with the least fat came from mice on the lower $CaCO_3$ diets, and those with the most from animals on the highest dietary $CaCO_3$ level.

The distribution of the fatty changes within the lobule was also studied. When the amount present was small, the fat was generally distributed fairly evenly throughout each lobule (Pl. 2, 1). On the other hand, greater amounts of fat were almost invariably situated in the centrilobular areas (Pl. 2, 2). In the most severe cases, nearly every parenchymatous cell was affected, the appearance being almost that of adipose tissue, relieved only by small patches around the portal vessels (Pl. 2, 3).

The essential lesion in the hepatic cells was fatty degeneration. The earliest changes were manifested in the characteristic 'foam-cells' (Pl. 2, 4), in which the cytoplasm was partly broken down and contained droplets of fat. In more advanced stages, the cells were swollen and contained large vacuoles; and in the most severe cases only the cell wall remained, sometimes with the remains of the nucleus close against it, the cytoplasm having been completely replaced by immense fat globules.

Bone marrow. All the bone-marrow specimens examined were highly cellular, with hyperplasia of the erythroid cell series. The reaction was most marked in marrows from the high $CaCO_3$ groups, which contained an abundance of immature red cells, particularly early and late normoblasts (Pl. 1, 3).

Experiment 2

The procedure described under Exp. 1 (see above) was adopted to assess the amount of visible fat in each of the 128 liver sections. Objectivity in assessment was attained by labelling each slide with a code number which gave the observer no clue to the

identity of the specimen, and by recording assessments by two independent observers. The summarized results are presented in Table 6.

Table 6. *Exp.* 2 (*first litters and mothers*). *Diet-group means and standard errors for quantity of visible liver fat, assessed* 1–4 *histologically, and the separate effects of the dietary supplements*

	First litters		Mothers	
Supplement†	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$
—	1·6	2·5	2·0	1·2
Fe	0·9	1·9	2·0	2·1
P	1·6	1·6	1·6	2·1
Fe+P	0·8	2·1	1·0	1·9
S.E. of difference between means of:				
Thirty-two animals	±0·26		±0·24	
Sixteen animals	±0·37		±0·35	
Eight animals	±0·53		±0·49	
Effect† of:				
$CaCO_3$	+0·81**		+0·19	
Fe	−0·44		0·00	
P	−0·19		−0·19	

† See footnotes to Table 4.

The effect of the $CaCO_3$ additions to the mother's diet was to increase the amount of visible fat in the livers of weanlings ($P < 0{\cdot}01$), and there was a suggestion that dietary Fe may have had the opposite effect. The effect of dietary P was not significant, nor were there any dietary effects on the livers of the mothers.

DISCUSSION AND CONCLUSIONS

In these experiments the main haematological findings were clear-cut: calcium carbonate, when added to the diet of breeding mice, induced a marked anaemia in both dams and litters. The immediate cause of the anaemia was undoubtedly an induced iron deficiency, and was evidenced by the hypochromic, anisocytic appearance of the erythrocytes in blood smears, the normoblastic reaction in the bone marrow and the quantitative haematological findings. The anaemia could be prevented by adding ferric citrate to the diet. The effects of the calcium carbonate could not, however, be neutralized merely by restoring the original Ca:P ratio of the diet with a supplement of sodium dihydrogen phosphate.

The anaemia in both weanlings and mothers was characterized by much reduced Hb, P.C.V. and M.C.H. values. The R.B.C. and M.C.H.C. were also decreased, markedly in the weanlings but less so in the dams. Only in the dams, however, did the supplements affect the M.C.V.; this was on the whole raised by Fe and lowered by $CaCO_3$, although the effects were uneven. The relative magnitudes of all effects may be compared in Fig. 1, which has been scaled so that for each attribute the standard errors are represented by equal positive and negative excursions from the base-line.

Discussing the haematology of iron-deficiency anaemia, Parsons (1938) states that, at least in rats, its course is characterized by three stages: there is at first a microcytosis

with accompanying mild polycythaemia, then the erythrocytes become hypochromic, and finally erythropenia develops. The syndrome seen in the dams presented all these and many of the other accepted characteristics of iron-deficiency anaemia (see, for example, Whitby & Britton, 1950). The picture was generally similar in the weanlings, except that microcytosis could not be demonstrated, and that changes in the other haematological attributes were more pronounced than in the mothers.

In this connexion, it must be remembered that, as was found by Bacharach, Cuthbertson & Thornton (1949), the effects of iron deficiency are more obvious in weanlings than in their dams. The basal diet in the present experiments contained

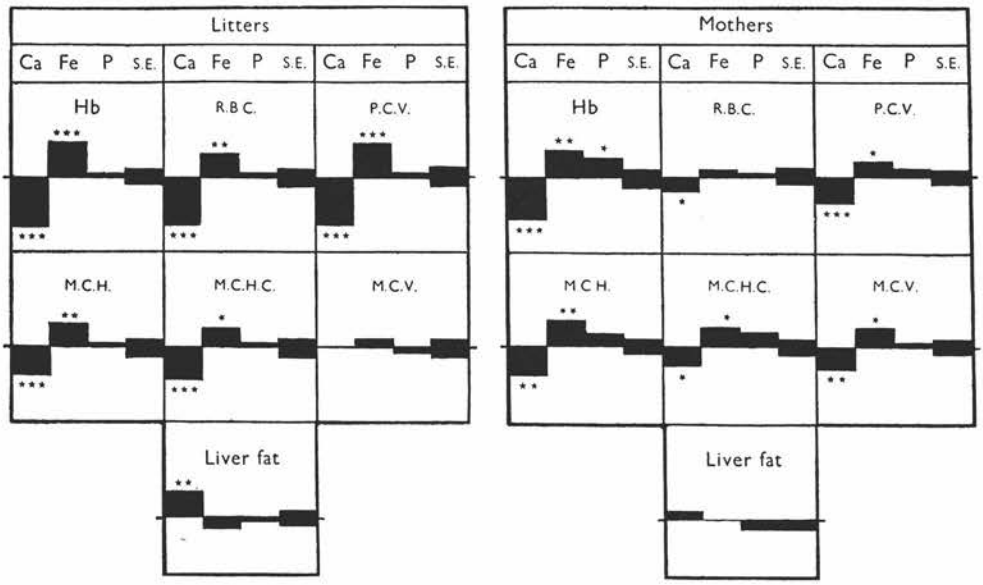

Fig. 1. Exp. 2. Overall independent effects of dietary supplements, to show the relative influence of each on various blood attributes and liver fat. The base-lines represent the overall mean measurements; positive effects (i.e. increases) above the base-line, negative effects (i.e. decreases) below the base-line. The diagram has been scaled so that the standard error is represented in each instance by the same positive and negative excursions from the base-line. Significance of effects is indicated by * ($P<0{\cdot}05$), ** ($P<0{\cdot}01$) and *** ($P<0{\cdot}001$). Ca = effect of 1·5 % supplement of calcium carbonate; Fe = effect of 10 p.p.m. supplement of iron as ferric citrate; P = effect of 0·57 % supplement of phosphorus as sodium dihydrogen phosphate; S.E. = standard error of the mean.

only 35 p.p.m. Fe (Richards & Greig, 1952), so that animals from all diet-groups were to some extent iron-deficient; indeed, anaemia of the hypochromic microcytic type was already established in litters from even the low-$CaCO_3$ groups, when compared with litters from several stock diets (unpublished observations). The relative differences between weanlings and mothers can therefore be explained on the grounds that the anaemia was much more severe in the weanlings, and consequently the first stage quoted above was not seen.

This explanation might also account for the failure to detect changes in the weanlings' M.C.V.; it would do so if microcytosis were either not progressive in iron-deficiency anaemia or were masked in severe cases by anisocytosis which, as pointed

out by Price-Jones (1932), is a feature of this condition. Certainly anisocytosis was well marked in these experiments (Pl. 1, 1). It may have been that a species peculiarity served to aggravate the anisocytosis: polychromatic cells, which are relatively larger than mature erythrocytes, are always numerous in normal mouse blood. Consequently, they may enter the circulation in greater numbers in response to the stress of severe anaemia, although in other species whose blood normally contains only fully haemoglobinized cells, such young cells are seldom mobilized. Although $CaCO_3$ did not reduce the M.C.V., it may not be unimportant that the effect of Fe, which was to raise the Hb and so, presumably, to lessen the anisocytosis, approached statistical significance in raising also the M.C.V. It is thus possible that there were no overall effects on the M.C.V. of the weanlings because macrocytosis in the young cells balanced microcytic changes in the mature erythrocytes.

On the other hand, the estimation of M.C.V. is always subject to considerable technical error. It is calculated from a formula which reflects the errors of two observed measurements; in particular, the notoriously inaccurate R.B.C. is the denominator in the formula, so that random errors in its measurement are greatly magnified in the resultant M.C.V. This is true, of course, for all values of R.B.C.; but when the R.B.C. is small (as it was so often in the anaemic weanlings), not only will errors of the same magnitude affect the M.C.V. to a proportionately greater degree, but substantial errors in its own measurement are more likely. Averages of many observations were taken, and no doubt these errors cancelled themselves out to some extent, but it was clear from the full data for both mothers and litters that variability in the recorded R.B.C. was greatest in the most anaemic animals. This means that much of the variability, and hence error, in the M.C.V. of the most anaemic animals can be ascribed to random variation in the R.B.C. Probably both anisocytosis and random error were jointly responsible for the irregularity observed in the weanlings' cell volume.

The outstanding histological feature was the increased amount of fat in the livers of weanlings from the high $CaCO_3$ groups. Whether this was a direct or an indirect effect of the supplement cannot be stated for certain, but it seems very likely that it was simply a reflexion of the severe anaemia, and was caused by local anoxia. This view is supported by the centrilobular distribution of the fat (areas farthest from the blood supply being most affected) and by degenerative changes in the other tissues, such as heart muscle and kidney tubules, which would probably be affected if the oxygen-carrying capacity of the blood were reduced. These changes did not, however, proceed to fatty degeneration.

Schmidt (1928), working with mice on iron-deficient diets, reported similar histological findings and also observed the associated hyperlipaemia. At the same time, he frequently found fatty deposits, which he regarded as infiltrations rather than degenerations, in the myocardium, pulmonary alveolar cells, and glomeruli of the kidneys, and also centres of calcification in many of the enlarged hearts. Such lesions were not seen in the present experiments, apart from one instance of calcification in the myocardium; but in Schmidt's animals the anaemia was more chronic.

It would seem, then, that the whole syndrome can be explained on the hypothesis that dietary $CaCO_3$ in some way interferes with the availability of Fe, as was postulated

by Richards & Greig (1952) for the effects of these diets on reproduction. The addition of phosphate, if it did not much help, certainly did not hinder Fe absorption. Previous work on this subject shows that there is a distinct division of opinion on the effects of dietary Ca salts, and the Ca:P ratio, on Fe absorption.

On the one hand, Kletzien (1935, 1938, 1940) found that, in both rats and mice, many Ca salts reduced Fe storage in the liver, whereas phosphates did not. Shelling & Josephs (1934) and Anderson, McDonough & Elvehjem (1940) also observed that Fe utilization was most efficient when the Ca:P ratio was low. Nordfeldt (1939) reported that calcium-lactate supplements increased the negative Fe balance of mature rats on a cow's milk diet, and Fuhr & Steenbock (1943) found that excessive dietary $CaCO_3$ reduced blood haemoglobin and Fe storage.

On the other hand, the view that low dietary Ca or high dietary phosphate will inhibit Fe absorption seems to have gained wide acceptance (McLester, 1927–49; McCance & Widdowson, 1944; Anonymous, 1950, 1951). It seems to date from the early experiments of von Wendt (1905) and Sherman (1907), and, although their methods were criticized by Davidson & Leitch (1933-4), there is also more recent evidence in its favour. Day & Stein (1938) reported that anaemia developed in rats fed on a high-P diet, unless Ca or some other phosphate-binding ion was also fed; Orten, Smith & Mendel (1936) also found that purified $CaCO_3$ prevented anaemia in rats fed on a diet low in inorganic salts, and Happ (1922) reported that rats on a low-Ca, high-P diet developed anaemia. Further support for this view comes from Kinney, Hegsted & Finch (1949) and Hegsted, Finch & Kinney (1949) who have shown that rats fed on low-P diets absorb excessive quantities of Fe. Again, many workers (e.g. Waltner, 1927; Cox, Dodds, Wigman & Murphy, 1931; Brock & Diamond, 1934; Deobald & Elvehjem, 1935) have demonstrated that excessive dietary Fe causes a negative P balance, presumably by forming an insoluble iron phosphate in the bowel and so preventing proper P utilization. Brock (1937) also showed that dietary Fe, while it increased faecal P, did not affect faecal Ca. It is not unreasonable to argue the converse—that high dietary phosphate will similarly interfere with iron availability—and thence to presume that dietary Ca will increase iron availability by precipitating the P as Ca phosphate.

There is thus considerable support for both opinions. The conflicting evidence, together with the findings reported in this paper, indicate that some other important factor, qualitative or quantitative, has not yet been defined.

SUMMARY

1. Weanlings born of white Swiss mice on diets containing 2 % added calcium carbonate were anaemic when compared with weanlings from diet-groups with 0 or 0·5 % added calcium carbonate. The dams on the high-calcium carbonate diets were also anaemic.

2. The addition to the diet of 10 p.p.m. iron as ferric citrate prevented the anaemia, but merely restoring the calcium:phosphorus ratio with sodium dihydrogen phosphate did not do so.

3. Second litters in all diet-groups were less anaemic than first litters.

4. The anaemia in both weanlings and dams presented the haematological features of iron-deficiency anaemia, except that the mean cell volume was not constantly reduced; possible reasons for this are discussed.

5. In association with the anaemia in the weanlings, there were fatty deposits in the liver, marked hyperlipaemia, and sometimes degenerative changes in other organs.

6. Consideration of the literature suggests that some unrecognized factor is important in dietary iron-calcium-phosphate relationships.

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EXPLANATION OF PLATES

PLATE 1

1. Blood films from weanling mice from high calcium-carbonate diet-groups, *a* stained Leishman, × 1800; *b* stained brilliant cresyl blue supravitally and Leishman, × 1500. Note the hypochromasia, anisocytosis and poikilocytosis, and, in *b*, the normoblasts and reticulocytes.
2. Heart muscle of weanling mouse from high calcium-carbonate diet-group. Note hypertrophy of the fibres and, in the lower half, degenerative changes. Haemalum and eosin, × 700.
3. Section of bone marrow of weanling mouse from high calcium-carbonate diet-group. Note the normoblastic hyperplasia. Haemalum and eosin, × 650.

PLATE 2

1, 2 and 3. Livers of weanling mice, containing varying amounts of fat (appears dark). Frozen sections, Scharlach R and haemalum. 1, × 108; 2, × 80; 3, × 95.

4. Liver of weanling mouse, to show the 'foam-cells' typical of the fatty degeneration. Haemalum and eosin, × 400.

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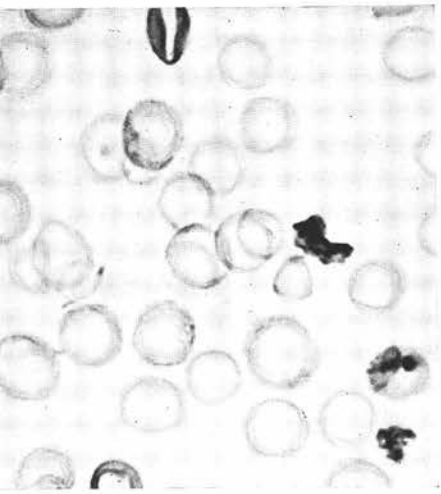
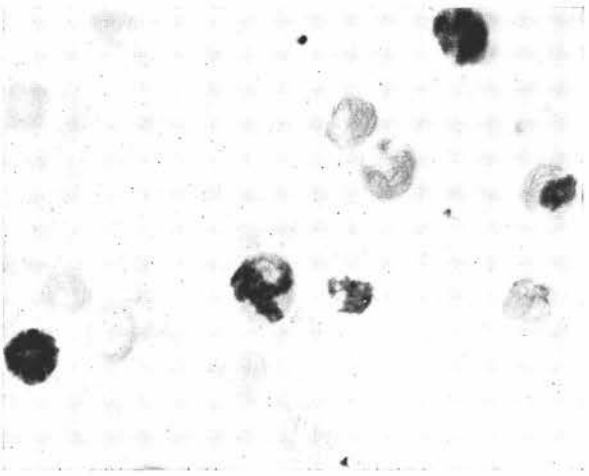


1 (*a*)



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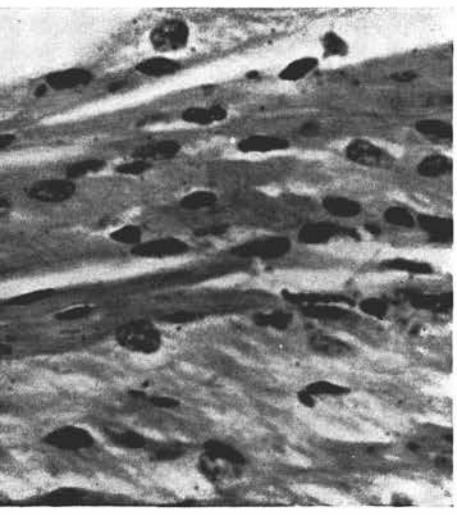

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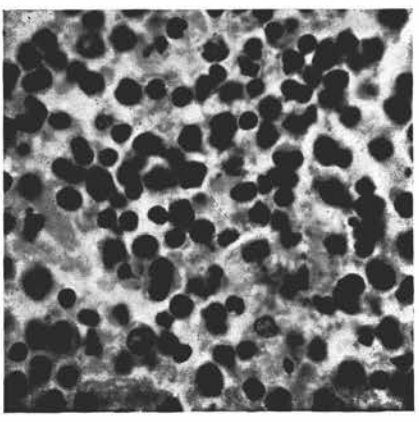

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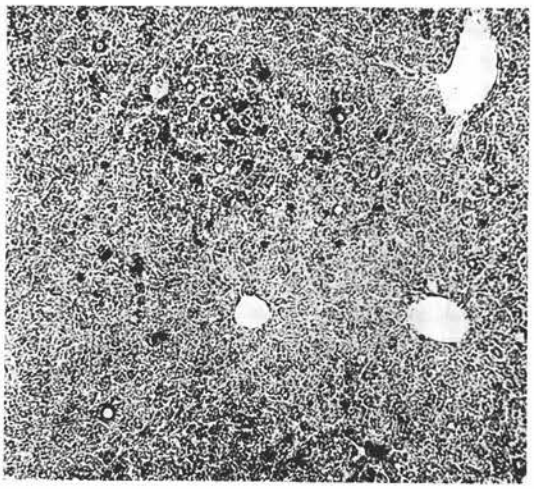

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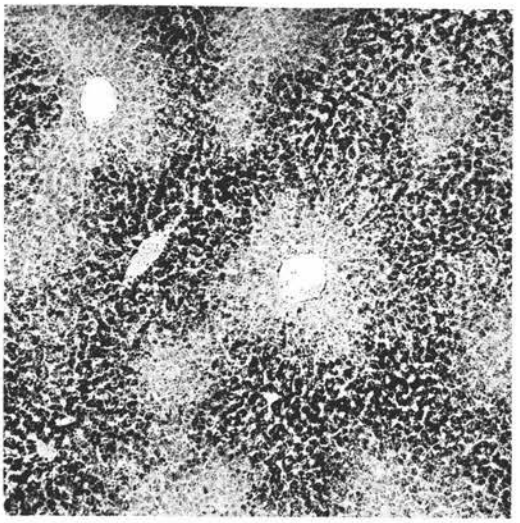

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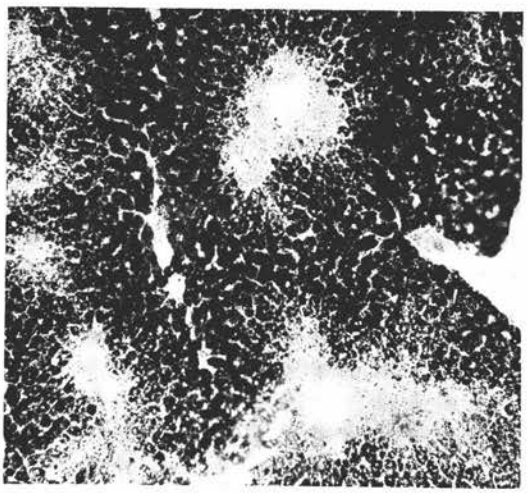

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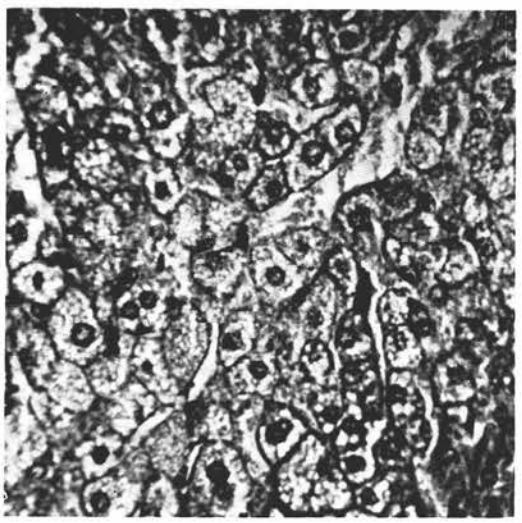

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